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## (54) Nutritional composition intended for specific gastro-intestinal maturation in premature mammals

(57) A nutritional enteral composition intended for favoring the growth and maturation of non-mature gastro-intestinal tracts of young mammals, which contains as a protein source a mixture of dietary protein hydrolysates and intact proteins being partly in the form of bioactive peptides.

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## Description

### Field of the Invention

**[0001]** This invention relates to an enteral composition containing peptides in an adapted profile size, bioactive peptides, intact proteins, and free amino acids intended for specific gastro-intestinal maturation in premature mammals.

### Background to the Invention

**[0002]** Nutritional compositions based upon hydrolysates of proteins such as milk or soy, are commonly used in infant and clinical nutrition and particularly in hypoallergenic formulas and formulas for patients suffering from various intestinal absorption problems. It is also known to use free amino acids in nutritional compositions for example for patients suffering from particular diseases or conditions such as inflammatory bowel disease, intractable diarrhoea, short bowel syndrome, and the like. Accordingly, amino acids are used either alone or in combination with protein or protein hydrolysates. Protein hydrolysates or free amino acid mixtures are also mainly used in particular cases such as allergy to whole proteins.

**[0003]** Another interest in using protein hydrolysates in nutrition is due to the fact that they are more rapidly absorbed in the intestine than whole protein or free amino acids. However, it is not clear whether this faster absorption translates into better nitrogen utilisation since studies carried out to date have provided conflicting results (Colin-Vidal *et al*; 1994; Endocrinol. Metab., 30, E 907-914). Further, this interest is in the sense of providing a source of amino acids to meet the general amino acids needs of the patient and not to specifically provide for the needs of individual gastro-intestinal maturation.

### Summary of the Invention

**[0004]** Accordingly, on one aspect, this invention provides a nutritional enteral composition intended for favoring the growth and maturation of non-mature gastro-intestinal tracts of young mammals, which contains as a protein source a mixture of dietary protein hydrolysates and intact proteins being partly in the form of bioactive peptides.

**[0005]** In this composition, the dietary protein hydrolysates are preferably in the form of a mixture of different size peptides, free amino acids or a mixture thereof. The dietary protein hydrolysates may be hydrolysates of animal proteins (such as milk proteins, meat proteins and egg proteins), or vegetable proteins (such as soy proteins, wheat proteins, rice proteins, and pea proteins). The preferred source is milk protein. The dietary protein hydrolysates can be used as such or like peptide fractions isolated from them.

**[0006]** The hydrolysed proteins may comprise at least 5 % (by weight, of the total protein content calculated as Nitrogen x 6.25) of hydrolysate having a degree of hydrolysis of about 40 and at least 5 % of hydrolysates having a lesser degree of hydrolysis. Free amino acids are preferably in an amount of about 0 to 20 % by weight of the total protein content (N x 6.25).

**[0007]** The intact proteins may be individual or enriched animal or vegetable protein fractions comprising whole milk, caseins, whey proteins, soy proteins or rice proteins, for example. They are preferably in an amount of at least about 5 % of the total protein content (N x 6.25).

**[0008]** The intact protein fraction may contain bioactive peptides such as TGF-β2 or a source of bioactive peptides such as beta-casein liberated in the gut by enzymatic hydrolysis. The final TGF-β2 concentration may be in the range of 0.1 to 4 ng/mg total protein, preferably about 1 to 2.5 ng/mg.

**[0009]** The nutritional composition may also contain a source of fat and a source of carbohydrates. This composition preferably contains a source of protein providing 5 to 30% of the total energy, a source of carbohydrates which provides 40 to 80% of the total energy, a source of lipids which provides 5 to 55% of the total energy, minerals and vitamins to meet daily requirements.

**[0010]** In another aspect, this invention provides the use of a selected mixture of dietary protein hydrolysates and intact proteins being partly in the form of bioactive peptides for the preparation of a nutritional enteral composition for favoring the growth and maturation of non- or pre-mature gastro-intestinal tracts of young mammals.

**[0011]** The nutritional composition also intends to cover very high nutrient needs for growth and development during that stage. It ensures optimal digestion and utilization (for tissue accretion) of the protein source and intends to minimize the nitrogen waste of the organism. Moreover, a mixture of intact protein, protein hydrolysates, bioactive peptides and free amino acids provides a better source of amino acids to meet the general amino acid needs of the patient in addition to specifically favor the maturation of individual organs.

**[0012]** Embodiments of the invention are now described by way of example only.

### Detailed Description of the Invention

**[0013]** In the specification, the term "degree of hydrolysis" (DH) means the percentage of nitrogen in the form of free alpha-amino nitrogen as compared to total nitrogen. It is a measure of the extent to which the protein has been hydrolysed.

**[0014]** The term bioactive peptide relates to i) a protein or peptide present as such in the preparation and demonstrating specific functional properties or ii) a protein or peptide containing an amino acid sequence with specific properties, this sequence being liberated in the gastro-intestinal tract during the natural process of di-

gestion.

**[0015]** According to a first aspect of the invention, the nutritional composition comprises as a source of protein a selected mixture of intact protein being partly in the form of bioactive peptides and dietary protein hydrolysates having a degree of hydrolysis in the range of about 5% to about 50% and free amino acids. The non-protein nitrogen concentration of the protein source can be comprised between 10% and 95% of the total nitrogen. Such protein source maximizes the area of the intestine in which the protein is digested and optimizes protein synthesis in the gut and peripheral tissues.

**[0016]** The nutritional composition can also contain a carbohydrate source, a fat source, vitamins and minerals.

**[0017]** The intact protein may be any suitable dietary protein; for example animal proteins (such as milk proteins, meat proteins and egg proteins); vegetable proteins (such as soy protein, wheat protein, rice protein, and pea protein); or combinations thereof. Milk proteins such as casein and whey protein are particularly preferred. They are preferably in an amount at least of about 5 % of the total protein content (calculated as Nitrogen x 6.25).

Dietary protein in the form of intact protein is found to increase the rate of muscle protein synthesis as compared to protein hydrolysates.

**[0018]** The dietary protein hydrolysates may come from any suitable dietary protein; for example animal proteins (such as milk proteins, meat proteins and egg proteins); vegetable proteins (such as soy protein, wheat protein, rice protein, and pea protein); or combinations thereof. Milk proteins such as casein and whey protein are particularly preferred. The hydrolysed dietary proteins may comprise at least 5 % (by weight, of the total protein content calculated as Nitrogen x 6.25) of hydrolysate having a degree of hydrolysis of about 40 and at least 5 % of hydrolysates having a lesser degree of hydrolysis.

In particular, hydrolysates having a degree of hydrolysis of about 10% to about 15%, are found to increase relative weight of the liver as compared to free amino acid mixes. Hydrolysates having a degree of hydrolysis of about 15% to about 25% are found to increase the concentration of protein in the jejunum, the relative weight of the jejunum and the rate of protein synthesis in the jejunum. Highly hydrolysed protein which has a degree of hydrolysis of greater than 25% or which contains more than 25% by weight of di- and tri-peptides, more preferably greater than 30%, is found to increase the rate of protein synthesis in the jejunum and the duodenum; particularly the duodenum.

**[0019]** The dietary protein hydrolysates may be produced using procedures which are well known in the art or may be obtained commercially. For example, nutritional formulas containing hydrolysates having a degree of hydrolysis less than about 15% are commercially available from Nestlé Nutrition Company under the trade mark Peptamen®. Hydrolysates having a degree of hydrolysis above about 15% may be prepared using the procedure described in EP 0322589.

**[0020]** The dietary protein hydrolysate source may also be in the form of a mix of free amino acids; preferably such that the mix provides a balanced amino acid profile. Free amino acids are preferably in an amount of about 0 to 20 % by weight of the total protein content (calculated as Nitrogen x 6.25).

Dietary protein in the form of a mix of free amino acids is found to increase the relative weight of the jejunum and the rate of protein synthesis in the jejunum.

**[0021]** The source of total proteins preferably provides about 5% to about 30% of the energy of the nutritional composition; for example about 10% to about 20% of the energy. The remaining energy of the nutritional composition may be provided in the form of carbohydrates and fats.

**[0022]** If the nutritional composition includes a fat source, the fat source preferably provides about 5% to about 55% of the energy of the nutritional composition; for example about 20% to about 50% of the energy. The lipids making up the fat source may be any suitable fat or fat mixture. Vegetable fats are particularly suitable; for example soy oil, palm oil, coconut oil, safflower oil, sunflower oil, corn oil, canola oil, lecithins, and the like. Animal fats such as milk fats may also be added if desired. The lipids may also include medium-chain triglycerides; for example up to about 60 % by weight of lipids as medium-chain triglycerides. Fractionated coconut oil is a suitable source of medium-chain triglycerides.

**[0023]** A source of carbohydrate may be added to the nutritional composition. It preferably provides about 40% to about 80% of the energy of the nutritional composition. Any suitable carbohydrates may be used, for example sucrose, lactose, glucose, fructose, corn syrup solids, and maltodextrins, and mixtures thereof.

**[0024]** Dietary fibre may also be added if desired. If used, it preferably comprises up to about 5% of the energy of the nutritional composition. The dietary fibre may be from any suitable origin, including for example soy, pea, oat, pectin, guar gum, and gum arabic.

**[0025]** Suitable vitamins and minerals may be included in the nutritional composition in an amount to meet the appropriate guidelines.

**[0026]** One or more food grade emulsifiers may be incorporated into the nutritional composition if desired; for example diacetyl tartaric acid esters of monodiglycerides, lecithin and mono- and di-glycerides. Similarly suitable salts and stabilisers may be included.

**[0027]** The nutritional composition is preferably enterally administrable; for example in the form of a powder, a liquid concentrate, a ready-to-drink, or a ready-to-administer beverage.

**[0028]** The nutritional composition may be prepared in any suitable manner. For example, it may be prepared by blending together the source of dietary protein, the carbohydrate source, and the fat source in appropriate

proportions. If used, the emulsifiers may be included in the blend. The vitamins and minerals may be added at this point but are usually added later to avoid thermal degradation. Any lipophilic vitamins, emulsifiers and the like may be dissolved into the fat source prior to blending. Water, preferably water which has been subjected to reverse osmosis, may then be mixed in to form a liquid mixture. The temperature of the water is conveniently about 50°C to about 80°C to aid dispersal of the ingredients. Commercially available liquefiers may be used to form the liquid mixture. The liquid mixture is then homogenised; for example in two stages.

**[0029]** The liquid mixture may then be thermally treated to reduce bacterial loads, by rapidly heating the liquid mixture to a temperature in the range of about 80°C to about 150°C for about 5 seconds to about 5 minutes, for example. This may be carried out by steam injection, autoclave or by heat exchanger; for example a plate heat exchanger.

**[0030]** Then, the liquid mixture may be cooled to about 60°C to about 85°C; for example by flash cooling. The liquid mixture may then be again homogenised; for example in two stages at about 7 MPa to about 40 MPa in the first stage and about 2 MPa to about 14 MPa in the second stage. The homogenised mixture may then be further cooled to add any heat sensitive components; such as vitamins and minerals. The pH and solids content of the homogenised mixture is conveniently standardised at this point.

**[0031]** If it is desired to produce a powdered nutritional composition, the homogenised mixture is transferred to a suitable drying apparatus such as a spray drier or freeze drier and converted to powder. The powder should have a moisture content of less than about 5% by weight.

**[0032]** If it is desired to produce a liquid composition, the homogenised mixture is preferably aseptically filled into suitable containers by pre-heating the homogenised mixture (for example to about 75 to 85°C) and then injecting steam into the homogenised mixture to raise the temperature to about 140 to 160°C; for example at about 150°C. The homogenised mixture may then be cooled, for example by flash cooling, to a temperature of about 75 to 85°C. The homogenised mixture may then be homogenised, further cooled to about room temperature and filled into containers. Suitable apparatus for carrying out aseptic filling of this nature is commercially available. The liquid composition may be in the form of a ready to feed composition having a solids content of about 10 to about 14% by weight or may be in the form of a concentrate; usually of solids content of about 20 to about 26% by weight. Flavours may be added to the liquid compositions so that the compositions are provided in the form of convenient, flavoursome, ready-to-drink beverages.

**[0033]** In another aspect, this invention provides a method for increasing protein concentration and synthesis in the small intestine, the method comprising administering to a pre-mature or non-mature mammal an effective amount of a nutritional composition containing a dietary protein hydrolysates having a degree of hydrolysis of less than 50 % and intact proteins being partly in the form of bioactive peptides. Further, the dietary protein hydrolysate preferably has a non-protein nitrogen concentration of at least about 85% of total nitrogen. Non protein nitrogen is defined as the nitrogen fraction not recovered as a precipitate after acidification.

**[0034]** Preferably, the method may be used to treat premature or non-mature young mammals to promote growth and maturation of the gastro-intestinal tract. Additionally, the method can also apply to situations encountered in clinical nutrition when alterations of the normal growth or turnover of the gut mucosa occur, e.g. after long term total parenteral nutrition or malnutrition.

**[0035]** The nutritional enteral composition also intends to cover very high nutrient needs for growth, development and maintenance during those situations. It ensures optimal digestion and utilization (for tissue accretion) of the protein source and intends to minimize the nitrogen waste of the organism. The composition may also be used for patients with gut mucosa damage.

**[0036]** The amount of the nutritional composition to be administered will vary depending upon the state of maturation or growth of the gut of the mammal.

**Example 1**

Whole protein

**[0037]** An amount of 5 kg of whey protein (obtained from Meggle GmbH under the trade name Globulal 80) is dispersed in demineralised water at 55°C to obtain protein concentration (N*6.38) of 10% by weight. The pH of the dispersion is adjusted by the addition of 190 g of calcium hydroxide and the dispersion is cooled to room temperature. The proteins are then dried by lyophilisation and packaged into metal cans.

**[0038]** The whole proteins have a degree of hydrolysis of about 4.41% and a non protein nitrogen concentration of about 1.1% on the basis of total nitrogen.

Hydrolysate 1

**[0039]** An amount of 6.25 kg of whey protein (obtained from Meggle GmbH) is dispersed in 50 litres of demineralised water at 55°C. The pH of the dispersion is adjusted to 8.2 by the addition of 1.8 litres of 2M $Ca(OH)_2$. The proteins are then hydrolysed using 30 g of trypsin (Salt free pancreatic trypsin which has an activity of 6.8 AU/g and a chymotrypsin content of less than 5% and which is obtainable from Novo Nordisk Ferment AG, Dittigen, Switzerland). The hydrolysis reaction is continued for 4 hours at 55°C. During the reaction, the pH is regulated to 7.4 by the addition of 1.6N NaOH and 0.4N KOH. The enzymes are then inactivated by heating the reaction

mixture to 80°C and holding the mixture at this temperature for about 5 minutes. The mixture is then cooled to 16°C. The hydrolysed proteins are then dried by lyophilisation and packaged into metal cans. The hydrolysate has a degree of hydrolysis of about 14% and a non protein nitrogen concentration of about 54.5% on the basis of total nitrogen.

Hydrolysate 2

**[0040]** An amount of 6.25 kg of whey protein (obtained from Meggle GmbH) is dispersed in 50 litres of demineralised water at 55°C. The pH of the dispersion is adjusted to 7.5 by the addition of 1.6 litres of 1M $Ca(OH)_2$ and 162 ml of a solution of 1.6M NaOH and 0.4M KOH. The proteins are then hydrolysed using 50 g of trypsin (obtainable from Novo Nordisk Ferment AG). The hydrolysis reaction is continued for 4 hours at 55°C. During the reaction, the pH is regulated to 7.4 by the addition of 1.6N NaOH and 0.4N KOH. The enzymes are then inactivated and non-hydrolysed protein is denatured, by heating the reaction mixture to 90°C and holding the mixture at this temperature for about 5 minutes.

**[0041]** The mixture is then cooled to 56°C and hydrolysed again for 1 hour using 50g of trypsin at 55°C. During the reaction, the pH is regulated to 7.4 by the addition of 1.6N NaOH and 0.4N KOH. The enzymes are then inactivated by heating the reaction mixture to 80°C and holding the mixture at this temperature for about 5 minutes. The mixture is then cooled to 16°C. The hydrolysed proteins are then dried by lyophilisation and packaged into metal cans.

**[0042]** The hydrolysate has a degree of hydrolysis of about 17.3% and a non protein nitrogen concentration of about 65.9% on the basis of total nitrogen.

Hydrolysate 3

**[0043]** An amount of 6.25 kg of whey protein (obtained from Meggle GmbH under the trade name Globulal 80) is dispersed in 50 litres of demineralised water at 55°C. The pH of the dispersion is adjusted to 7.5 by the addition of 1.6 litres of 1M $Ca(OH)_2$ and 162 ml of a solution of 1.6M NaOH and 0.4M KOH. The proteins are then hydrolysed using 250 g of Alcalase 2.4L (EC 940459 - obtainable from Novo Nordisk Ferment AG). The hydrolysis reaction is continued for 4 hours at 55°C. For the first hour of the reaction, the pH is regulated to 7.6 by the addition of 1.6N NaOH and 0.4N KOH.

**[0044]** An amount of 250g of Neutrase 0.5L (obtainable from Novo Nordisk Ferment AG) is added and the proteins are further hydrolysed for 4 hours at 50°C. The enzymes are then inactivated by heating the reaction mixture to 90°C and holding the mixture at this temperature for about 5 minutes. The reaction mixture is then cooled to 55°C.

**[0045]** The pH of the reaction mixture is adjusted to 7.33 by the addition of 1.6N NaOH and 0.4N KOH and the reaction mixture hydrolysed again for 4 hours using 100g of pancreatin at 55°C. During the reaction, the pH is regulated to 7.5 by the addition of 1M NaOH. The enzymes are then inactivated by heating the reaction mixture to 90°C and holding the mixture at this temperature for about 5 minutes. The mixture is then cooled to 4°C. The hydrolysed proteins are then dried by lyophilisation and packaged into metal cans.

**[0046]** The hydrolysate has a degree of hydrolysis of about 35% and a non protein nitrogen concentration of about 92.6% on the basis of total nitrogen.

**Example 2**

**[0047]** In order to obtain a nutritionnal composition intended for specific gastro intestinal maturation in premature mammals, the following mixture is prepared :

i) 14.5 g/ 100 g powder total protein content:

10 % hydrolysate 2 as prepared in example 1,
40 % hydrolysate 3 as prepared in example 1,
50 % intact proteins (containing 1 ppm TGFβ2),

ii) 26 g/ 100 g powder of fat:

40 % medium chain triglycerides
60 % long chain triglycerides

iii) 53.6 g/ 100 g powder carbohydrates

65 % lactose
35 % maltodextrins

iv) and vitamins, minerals to meet daily requirements.

**Claims**

1. A nutritional enteral composition intended for favoring the growth and maturation of non-mature gastro-intestinal tracts of young mammals, which contains as a protein source a mixture of dietary protein hydrolysates and intact proteins being partly in the form of bioactive peptides.

2. A composition according to claim 1, wherein the dietary protein hydrolysates are in the form of a mixture of different size peptides, free amino acids or a mixture thereof.

3. A composition according to claim 2, wherein the dietary protein hydrolysates contain at least about 5 % (by weight, of the total protein content calculated as Nitrogen x 6.25) of hydrolysate having a degree of hydrolysis of about 40 and at least about 5 % of hydrolysates having a lesser degree of hydrolysis.

4. A composition according to claims 2 or 3, wherein free amino acids are in an amount of about 0 to about 20 % by weight of the total protein content (calculated as Nitrogen x 6.25).

5. A composition according to any of claims 1 to 4, wherein the intact proteins are in an amount of at least about 5% by weight of the total protein content (calculated as Nx6.25).

6. A composition according to any of claims 1 to 5, wherein the intact proteins are milk proteins, whey proteins, caseins and bioactive peptides such as TGF-β2.

7. A composition according to any of claims 1 to 6, wherein bioactive peptides represent at least about 0.1 to about 4 ng/mg total protein.

8. A composition according to any of claims 1 to 7 which contains a source of protein providing 5 to 30% of the total energy, a source of carbohydrates which provides 40 to 80% of the total energy, a source of lipids which provides 5 to 55% of the total energy, minerals and vitamins to meet daily requirements.

9. Use of a selected mixture of dietary protein hydrolyeates and intact proteins being partly in the form of bioactive peptides as protein source in the preparation of a nutritional enteral composition intended for favoring the growth and maturation of non-mature gastro-intestinal tracts of young mammals.

10. Use according to claim 9, wherein the dietary protein hydrolysates are in the form of a mixture of different size peptides, free amino acids or a mixture thereof.

11. Use according to claim 9 or 10, wherein the dietary protein hydrolysates comprise at least 5 % (by weight, of the total protein content calculated as Nitrogen x 6.25) of hydrolysate having a degree of hydrolysis of about 40 and at least 5 % of hydrolysates having a lesser degree of hydrolysis.

12. Use according to any of claim 9 to 11, wherein free amino acids are in an amount of about 0 to about 20 % by weight of the total protein content (N x 6.25)

13. Use according to any of claims 9 to 12, wherein the intact proteins are in an amount of at least about 5% of the total protein content.

14. Use according to any of claims 9 to 13, wherein the the intact proteins are milk proteins, whey proteins, caseins and bioactive peptides such as TGF-β2.

15. Use according to any of claims 9 to 14, wherein bioactive peptides represent about 0.1 to about 4 ng/mg total protein.

16. Use according to any of claims 9 to 15, in which the nutritional composition contains a source of protein providing 5 to 30% of the total energy, a source of carbohydrates which provides 40 to 80% of the total energy, a source of lipids which provides 5 to 55% of the total energy, minerals and vitamins to meet daily requirements.

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The present search report has been drawn up for all claims

| Place of search | Date of completion of the search | Examiner |
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## (54) Cysteine/glycine rich peptides

(57) Described is a method for the preparation of a mixture of peptides having a cysteine content between 7-20 w/w % from a protein source, comprising cysteine containing proteins, comprising the steps of:

a) cleaving the proteins of the protein source into peptides;
b) digesting the peptides obtained in step a) by an exopeptidase, the action of which is at least attenuated at the position of a cysteine in the peptide, therewith forming digested peptides having a terminal cysteine;
c) purifying the digested peptides,

and the use of the preparation as active component in a medicament, especially for the treatment of conditions mediated by oxidative damage and for the elevation of cellular glutathion levels in the human or animal body.

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## Description

**[0001]** The invention relates to a method for the preparation of a mixture of peptides having a cysteine- or cysteine/glycine content between 7-20 w/w %, to preparations comprising said peptides and to the use of such preparations as active compound in a medicament.

**[0002]** Peptides are defined as amino acid chains, derived from a protein; the molecular weight of the peptides is preferably between 200D and 8000D, more preferably between 1000D and 5000D.

**[0003]** In the art, there is a great demand for cysteine and cysteine/glycine comprising compounds for effective administration of said amino acids to the human or animal body. The availability of especially cysteine and to a lesser extent glycine, is a limiting factor in the syntheses of glutathion. Proper administration of cysteine, but also of glycine is therefore demanded in cases where an elevation of cellular glutathion levels in the human or animal body are needed.

**[0004]** Glutathion (GSH) is a tripeptide-thiol (L-γ-glutamyl-L-cysteinylglycine) having a broad range of vital functions, including protection of cells against oxygen intermediates, free radicals, byproducts of the oxygen requiring metabolism, and detoxification of xenobiotics. Further, glutathion seems to play a role in the prevention of cataract and oxidative DNA injury. Glutathion is therefore regarded as an important compound against oxidative stress related diseases like myocardial ischemia, cancer and cataract.

**[0005]** In view of the crucial role played by glutathion either in combatting the assaults of free radical injuries or in detoxification of xenobiotics, inclusing drug metabolites (such as cyclophosphamide, paraquat and acetaminophen) and in preventing peroxidation of cell components, a method for maintaining hepatic stores of glutathion, particularly during times of stress to the body, including chemotherapy, is needed.

**[0006]** In the art, various methods are known to increase cellular levels of glutathion. Administration to animals of the glutathion amino acid precursors glutamic acid, cysteine and glycine, may produce an increase in cellular glutathion, but there is a limit to the effectiveness of this procedure.

**[0007]** Cellular concentrations of GSH are dependent on the supply of cysteine, which is often the limiting amino acid, and which is derived from dietary protein and also by trans-sulfuration from methionine in the liver. However, administration of cysteine as free amino acid is not an ideal way to increase GSH concentrations because cysteine is rapidly metabolised and furthermore, appears to be toxic to cells at higher concentrations. Administration to animals of compounds that are transported into cells and converted intracellularly into cysteine is sometimes useful in increasing cellular glutathion levels.

**[0008]** Another way in which tissue GSH concentration may be increased is by administration of gamma glutamyl-cysteine or of gamma-glutamylcystine. The administered gamma-glutamyl amino acid is transported intact and serves as a substrate of GSH synthetase. It is also known that administration of N-acetyl-L-cysteine can often increase tissue concentrations of GSH. Other reports on using N-mercaptopropionyl glycine for increasing intracellular glutahion are known. A few clinical trials have been done using mercaptopropionyl glycine to elevate intracellular glutathion.

**[0009]** That the administration of glutathion itself might lead to increased glutathion levels has also been considered. However, there is no published evidence that shows that intact glutathion enters cells. In fact, there are several reports on particular biological systems indicating that glutathion itself is not transported into cells. The increase in cellular glutathion sometimes found after administration of glutathion is due to (a) extracellular breakdown of glutathion, (b) transport into cells of free amino acids or dipeptides derived from glutathion extracellularly, and (c) intracellular resynthesis of glutathion.

**[0010]** Apart from these conventional methods for increasing glutathion levels, there have been several attempts to demonstrate how glutathion can be enhanced intracellularly. All these relate to synthetic derivatives or about intact undenatured proteins which are heat labile and none whatsoever to natural derived peptide mixtures. Some of the relevant ones are summarised below:

**[0011]** US Patent No 5,869,456 relates to preparation of pure alkyl esters of glutathion (95% pure) and a method for increasing intracellular glutathion levels by administering such alkyl diester of glutathion.

**[0012]** US patent 5,464,825 describes the method for preparation and use of N-acyl glutathion monoalkyl esters to provide increased intracellular levels of glutathion or glutathion equivalents, e.g. N-acyl glutathion or glutathion monoalkyl esters.

**[0013]** US patent 5,248,697 describes a method for maintaining and/or enhancing tissue or plasma levels of glutathion. The patent teaches the art of treatment of a mammal with a supranormal amount of glutamine, or a glutamine equivalent, to prevent the reduction in tissue glutathion levels associated with exposure of the mammal to a compound capable of oxidative injury to the tissue.

**[0014]** US patent 4,665,082 discusses the role of L-2-oxothiazolidine-4-carboxylate, a sulfur analog of 5-oxoproline, cleaved by the enzyme-5-oxo-L-prolinase to form cysteine, thus providing the basis for a cysteine delivery system by the addition of L-2-oxothiazolidine-4-carboxylate to base amino acid solutions or by injecting it directly into in vivo cells.

**[0015]** DE patent No 4,329,857 teaches the use of thiol compounds (cysteine and its derivatives or analogues like N-acetyl cysteine, homocysteine, glutathion, 2-oxothiazolidine-4-carboxylic acid) as an agent for strengthening the

immune system and immune reactions.

**[0016]** According to the present invention, a novel method for the preparation of a mixture of peptides having a cysteine content between 7-20 w/w% from a protein source, comprising cysteine containing proteins is provided. The protein source is preferably a natural protein source. The peptide mixture prepared according to this embodiment of the present invention has the advantage that it is derived from natural protein sources and will not show any adverse side-effects, whereas chemically produced cystein derivatives as mentioned in the prior art, have shown adverse side effects. There has been found that such a preparation of a peptide mixture can be very advantageously used as cysteine source in diet supplements or in medicaments, as will be explained below.

**[0017]** The method is characterized in that it comprises the steps of:

a) cleaving the proteins of the protein source into peptides;
b) digesting the peptides obtained in step a) by at least one exopeptidase, the action of which is at least attenuated at the position of a cysteine in the peptide, therewith forming digested peptides having a terminal cysteine;
c) purifying the digested peptides.

**[0018]** In the first step a) proteins of the protein source are cleaved into smaller peptides. This cleavage can be performed by cleavage reactions, known in the art; preferably, the cleavage is performed by enzymatic hydrolysis of the peptide bonds of the protein by e.g. an endopeptidase, resulting in the peptides of about the desired length, and therewith increasing the amount of substrate for the exopeptidase. In a second step, the peptides as obtained by the cleavage reaction, are digested by at least one exopeptidase. With "at least one exopeptidase" is meant that the digestion reaction can be carried out by one or more different exopeptidases. Exopeptidases release amino acids from the terminal ends of the peptides one by one. The exopeptidase and the digestion reaction conditions are chosen such, that the exopeptidase action is at least attenuated at the position of a cysteine in the peptide. With "at least attenuated" is meant that the exopeptidase does not remove the cysteine from the peptide at the chosen reaction conditions or has very low preference for the cleavage of cysteine, therewith rendering said cleavage reaction very slow compared to cleavage of other amino acids from the peptide. By the use of such an exopeptidase and condition, the peptides are generated of which the terminal amino acids have been removed up to the cysteine residue most close to said terminus. The skilled person will be able to find conditions at which commercially available enzymes with exopeptidase function having attenuated action at the cysteine. It is to be understood that the peptides may have one or more amino acid chains that are coupled to each other by disulfide bridges of cysteine residues, present in the said amino acid chains. "A digested peptide having a terminal cysteine" therefore reflects to the fact that at least one of the termini of such a multi-chain peptide has a terminal cysteine. Of course, such a peptide may contain more than one terminal cysteine. Preferably, the enzymatic activity is inactivated before the purification step, e.g. by a pH shift or a thermal heat inactivation treatment.

**[0019]** Preferably, the exopeptidase comprises Carboxypeptidase Y (E.C.3.4.16.1.), as it has been found that this enzyme can be very effectively attenuated at cysteine residues, therewith producing peptides with terminal cysteine residues.

**[0020]** The cleavage step a) and the digestion step b) can be conducted simultaneously, e.g. by using an endopeptidase and an exopeptidase that both function at the same reaction conditions. Also, enzyme preparations can be used that have both endopeptidase and exopeptidase activity.

**[0021]** Finally, these digested peptides are purified. Suitable methods to purify the digested peptides from free amino acids, released by the exopeptidase, are known in the art. Since a difference in molecular weight is created between the cystine and glycine containing peptides and the other free amino acids, the cystine and glycine peptides can be purified using this difference. Several techniques, known in the art, could be used. Preferably the free amino acids are separated using a membrane process, preferably ultra or nanofiltration. The purification step can also advantageously comprise the use of an immobilized metal affinity chromatography step (IMAC) accordingly to Kronina et al., Journal of Chromatography A, 852 (1999) pp 261-272. The cysteine and glycine rich peptides can hereafter be dried.

**[0022]** In a special embodiment, the exopeptidase in step b) and the cleavage reaction are chosen such, that the exopeptidase is at least attenuated both at the position of a cysteine as well as of glycine in the peptide. This will result in digested peptides having predominantly a terminal cysteine or glycine.

**[0023]** The purified peptides, either enriched in cysteine residues or enriched in both cysteine and glycine residues, have shown to be very suitable sources for these limiting amino acids to be readily administered, in order to elevate the cysteine and glycine rates in the human or animal body, and may therefore elevate the intracellular glutathion levels.

**[0024]** The protein source may be any source as long as it comprises cysteine-containing proteins. In case a cysteine and glycine rich peptide preparation is to be produced, the protein source should contain proteins that contain glycine and cysteine.

**[0025]** Preferably, the protein source comprises at least two different proteins, that both contribute to the glycine and/or cysteine content of the peptides. One of the proteins may be glycine rich, whereas the second protein may be cystein

rich. The protein source can also be prepared before being subjected to the method of the present invention, by e.g. two or more protein sources before or during the cleavage step.

**[0026]** Preferably, the protein source consists of edible proteins, so that the digested peptides can be used as food additive. In a very special embodiment, the protein source comprises whey protein isolates (WPI) and/or whey protein concentrates (WPC). The terms "whey protein isolates" and "whey protein concentrates" are known in the field. Whey protein cincentrate is a whey protein product having 35-80 w/w% protein, whereas whey protein isolate has a protein content of 90 w/w% or higher. An example of WPC is Esprion 580 from DMV International; an example of WPI is Bipro from Bio-isolates Ltd. Whey protein is an important cysteine source and it is thought that whey protein concentrate induces glutathion production in animal organs, see e.g. US-5 451 412. However, whey protein concentrates as such are not as suitable for the elevation of the intracellular glutathion levels compared to the peptides according to the present invention. The concentration of cystein and glycine in the intact whey proteins is much lower than in the peptides of the invention, and therefore requires much higher doses of the intact whey protein to reach an acceptable level of cystein in the application.

**[0027]** A further disadvantage of US 5 451 412 is that the use of totally undenatured whey protein products can be very costly since it requires very delicate process conditions. Whey protein isolate comprises very suitable cysteine and glycine rich proteins, such as albumin, especially αlactalbumin and bovine serum albumin. Said proteins are advantageously used in or as starting protein source of the method according to the invention.

**[0028]** In another preferred embodiment, the protein source comprises one or more of the group consisting of albumine, especially αlactalbumin, bovine serum albumin, egg proteins (e.g. ovalbumin, cystatin) wheat gluten, maize protein isolate.

**[0029]** Preferably, steps a) and b) are done at conditions, wherein sulfur bridges between cysteine residues as present in the proteins in the protein source are kept in the oxidised form as much as possible. In this way, cysteine-rich peptide mixtures are obtained, in which most of the cystein residues are oxidised and coupled to other peptides through disulfide bridges. Although the correct nomenclature for cysteins in oxidized form (i.e. being coupled to another cysteine residu by a sulfur bridge) is "cystine", in this application "cysteine" is defined both as cysteine in the reduced form (having free SH-groups) as in the oxidized (cystine) form. Peptides, wherein the sulfur bridges between the cysteine residues are intact, may mimick parts of the native original protein from which the peptides are derived, therewith possibly conferring an improved biologic action compared to that of the separate peptides in reduced form. Further, the oxidized form is less reactive and therefor more stable in applications that undergo a heat treatment like pasteurization or sterilization.

**[0030]** A further advantage is the fact that many enzymes having exopeptidase activity do not cleave oxidized cysteines, whereas cysteines in reduced form may be cleaved by said enzymes from the peptides, although with a relative low activity. In order to produce peptide mixtures in native, i.e. undenatured form, steps a) and b) are preferably done at a pH between 2 and 8.

**[0031]** It is preferred to carry out the hydrolytic processes in acidic environments. At acid pH the disulfide bridges in cystine are more stable than at basic pH. [Creighton, T.E., 1993, Proteins: structures and Molecular Properties. 2nd Ed.; Freeman and Company, New York]

**[0032]** It is preferred to cleave the proteins of the protein source in step a) by an enzyme with endopeptidase function. Using such an enzyme makes it possible to cleave the proteins under undenaturing (i.e. native) conditions, resulting in undenatured cleavage products. Physical or chemical cleavage mostly implicates application of denaturing conditions that can not be used if intact native peptide mixtures are to be obtained. For this, the expopeptidase digestion should also preferably take place at undenaturing conditions. The skilled person will know the proper conditions to yield intact native peptide mixtures. "Intact native peptide" is in this content to be understood as a peptide, having the same conformation as the said peptide has in the native, functional protein.

**[0033]** In a very attractive embodiment, the enzyme with endopeptidase function also has exopeptidase function, the exopeptidase function of which is attenuated at the position of cysteine or both at glycine and cysteine. Such enzymes are known in the art and the advantage thereof is that steps a) and b) can be done simultaneously. Examples of preferred enzymes having both endopeptidase as exopeptidase functions are Flavourzyme, Acid Protease A, Protease M, Protease 2A, Protease B, Corolase PN-L, Acid Protease or a combination of one or more thereof.

**[0034]** The invention further relates to preparations comprising cysteine-rich peptides, comprising 7-20 w/w % cysteine and to such a preparation comprising 7-20 w/w % of cysteine/glycine. As indicated above, said preparations can advantageously be used for administration to animals or humans in order to effectively improve the cysteine uptake of cysteine or a combination of cysteine and glycine for e.g. elevation of the intracellular glutathion level. Preferably, at least 80% of the peptides of the preparation comprises terminal cysteines and/or glycines, which are then readily available for the human or animal body. These terminal cysteines and/or glycines are obtained by the use of the exopeptidase as discussed above.

**[0035]** Further, the invention relates to the use of a preparation according to the invention as active compound in a medicament, especially in a medicament for treatment of conditions mediated by oxidative damage and in a medica-

ment for the elevation of cellular glutathion levels in the human or animal body. For this, the preparation can be combined with any suitable carrier, diluent adjuvant etc. in order to obtain the medicament in the desired administration form. The preparation can also advantageously be used in an infant formula, e.g. in a breast milk substitute.

**[0036]** The invention is now illustrated in the following examples and figures which are meant to be illustrative only and not to limit the scope of the invention.

Fig. 1a shows an absorption spectrum at 214 nm of a hydrolysate according to the invention,

Fig. 1b shows a fluorescence spectrum with an excitation wavelength of 386 nm and an emission wavelength of 514 nm of the hydrolysate of fig. 1a.

Example 1

**[0037]** A 10% whey protein isolate (WPI) solution is prepared and then hydrolysed using enzymes. Several combinations of enzymes were used (Table 1).

Table 1:

| Enzyme(s) used | | |
|---|---|---|
| Exp No | Enzyme 1 | Enzyme 2 |
| A | Pepsin (Merck) 0.5% | Protease M (Amano) 0.5% |
| B | Pepsin (Merck) 0.75% | Corolase LAP (Rohm) 2% |
| C | Pepsin (Merck) 0.5% | Acid Protease (EDC) 0.5% |
| D | Flavourzyme (Novo) 1% | |
| E | Acid Protease (EDC) 1% | |

**[0038]** Solutions 1 - 3 were first hydrolysed with pepsin for 6 hours at pH 2.0. Hereafter, the pH was increased to 7.0 using sodium hydroxide. The second enzyme was added and solutions incubated for 20 hours. The solutions containing a single enzyme were hydrolysed for 20 hours at 50°C at pH 7 and 3 for respectively Flavourzyme and Acid Protease. Hydrolytic reaction was stopped by heating the solutions to 85°C for 15 minutes. Hereafter, the free amino acids were removed from the peptides containing cysteine using ultrafiltration. A membrane with a nominal molecular weight (NMW) cut off of 1000 dalton was used. The solutions were ultrafiltered to 500% diafiltration.

Protein was measured using the Kjeldahl method. Cysteine concentration was measured using the Ellmann's reagents. [Beveridge et al (1974) Journal of Food Science Volume 39, p. 49 - 51]

The peptides were then freeze dried.

**[0039]** The table below lists the concentrations of both cystine and glycine in the whey protein isolate and peptides.

| | Total Cystine on protein | Total Glycine on protein |
|---|---|---|
| WPI | 3.3% | 1.9% |
| 1 | 8.5% | 2.4% |
| 2 | 11.9% | 2.7% |
| 3 | 12.4% | 2.7% |
| 4 | 9.7% | 2.1% |
| 5 | 10.9% | 3.0% |

Example 2

**[0040]** A 10% whey protein concentrate containing 80% protein solution is prepared and then hydrolysed using 1% Acid Protease form Enzyme Development Corporation. The solution was hydrolysed for 20 hours at pH 3.0. The reaction was stopped by heating the solution to 90°C for 10 minutes. Hereafter, the solution was ultrafiltered using a membrane having a NMW cut off of 1000 dalton.

Cysteine concentration was measured as a function of the %-diafiltration (table 2).

Table 2:

| Cysteine concentration as a function of the %-diafiltration | | | |
|---|---|---|---|
| | Weight (g) | Protein (%) | Cysteine (%) |
| Hydrolysate | 172 | 71.5 | 2.1 |
| 100 % diafiltered permeate | 167 | 68.0 | 0.2 |
| 200% diafiltered permeate | 150 | 71.3 | 0.1 |
| Retentate after diafiltration | 75.0 | 71.4 | 4.1 |

Example 3

**[0041]** 100 lt of a 5% whey protein isolate solution is prepared and then hydrolysed using 2% Acid Protease form Enzyme Development Corporation. The solution was hydrolysed for 12 hours at pH 3.0. The reaction was stopped by heating the solution to 80°C for 30 minutes. Hereafter, the solution was ultrafiltered on a pilot UF unit using Koch HFK 328 membrane having a NMW cut off of 5000 dalton. The hydrolysate was split in two parts. one part was filtered at the pH as is (3.8). The pH of the other part was first raised to 7.0 using sodium hydroxide after which it was ultrafiltered.

**[0042]** Cysteine concentration was measured as a function of the pH during ultrafiltration (table 3).

Table 3:

| Cysteine concentration as a function of the pH during ultrafiltration | |
|---|---|
| Sample | Cysteine on dry matter |
| Hydrolysate | 3.14 |
| Retentate UF pH 3.9 | 7.73 |
| Retentate UF pH 7.0 | 8.14 |

Example 4

**[0043]** The hydrolysate as in example 5 was nanofiltered using the Celgard NF-PES-10 membrane having a NMW cut off of dalton.
The NF-conditions were:

| | |
|---|---|
| Pressure | 30 bar |
| Temperature | 50-55°C |
| Initial flux | 58 ltrs/m$^2$/hr |
| End flux | 23 ltrs/m$^2$/hr |
| Process | concentrated to 30 ltrs and then 200% diafiltration |

**[0044]** The resulting peptides in the retentate contained 12.9% Cysteine and 3.1% Glycine.

Example 5 HPLC specific for Cys Peptides

**[0045]** A Reversed Phase HPLC-method (RPC) was set-up to identify and quantitate cysteine containing peptides in a mixture of peptides. The cysteine residues were first labelled with a fluorescent label (SBD-F; 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid; Sigma F-4383). This label specifically binds to cysteine residues.
In total 300 µl sample (100µg protein per ml), 600 µl incubationbuffer (250mM borate buffer, pH 8, 5mM EDTA), 300 µl fluorescent probe (0,1 % (W/V) in water), 297 µl $H_2O$ and 3 µl TBP (tributylfosfine, Fluka) are pipetted in a vial. The vial is capped, the mixture mixed well and incubated at 60C for 10 minutes. The final concentration of the sample is 0.02 mg/ml.
Hereafter the mixture is cooled to room temperature by putting on ice. Solution is filtered using 0.45µm PVDF filter (millipore, Millex-HV).
The filtered solutions are analysed by reversed phase chromatography using a Widepore C18 5µm RPC column (Bak-

er). The binding buffer consisted of demineralised water/0.1 % TFA (trifluorazijnzuur) and the peptides were eluted using a acetonitril/0.083% TFA buffer (buffer B). The level of Buffer B was increased to 60% in 90 minutes, whereafter tightly bound material was removed by running 100% buffer for 20 minutes. The injection volume was 150µl sample. The peptides are detected by measuring absorption at 214nm and fluorescence (excitation and emission wavelengths respectively 386nm and 514nm), see fig.1.

**[0046]** Upper panel of fig.1 shows the hydrolysate from example 1 before separation detecting the peptides by measuring adsorption at 214nm Lower panel shows the same hydrolysate detecting specific cysteine containing peptides measuring the fluorescence.

Example 6

**[0047]** The cysteine and glycine rich peptides can be used in clinical enteral nutrition formulas. A recipe for such formula is as follows:

| | |
|---|---|
| Cys and Gly rich peptides | 5.00% |
| Calcium Caseinate (DMV International) | 1.96% |
| Malto dextrin DE-20 | 14.0 % |
| Emulsifyer (Stemphil E60; Stern) | 0.30% |
| Oil-mix (50% sunflower; 20% MCT; 30% soy-oil) | 4.90% |
| Sodium Chloride | 0.09% |
| Tri-Calcium Phosphate | 0.95% |
| Magnesium Chloride | 0.15% |
| Calcium dl-Hydrogen Phosphate | 0.13% |
| Tri-Sodium Citrate | 0.086% |
| Water | 77.99% |
| Total | 100% |

**[0048]** Caseinate is dissolved in part of the demineralised water at 60°C; emulsifier is dissolved in the oil-mix; salts are dissolved in 75 ml water. Hereafter, the oil mix, salt, malto dextrin solution and residual water are subsequently mixed in the caseinate solution. This mixture is homogenised twice at 350 bar and at 70°C.
The Cysteine & Glycine rich peptides are then dissolved in the emulsion. The pH is adjusted to 7.0 - 7.1 using sodium hydroxyde and then the product is retort sterilised for 10 minutes at 121°C.

Example 7

**[0049]** The peptides can be used in infant formula's. A model recipe is as follows:

| Component | Concentration (g/lt) |
|---|---|
| Cys and Gly rich peptides | 10.0 |
| WE80BG (whey protein hydrolysate DMV International) | 10.0 |
| Edible Lactose (DMV International) | 30.0 |
| Malto dextrin DE-20 | 23.0 |
| Corn Syrup Solids | 25.0 |
| Emulsifier (Stemphil E60; Stern) | 5.0 |
| Oil-mix (45% sunflower; 25% MCT; 30% soy-oil) | 40.0 |
| Calcium ortho phosphate | 1.8 |
| Calcium carbonate | 1.3 |
| Magnesium Chloride | 0.3 |
| Potassium Chloride | 0.4 |
| Tri-Sodium Citrate | 0.5 |
| Water | 852.7 |
| Total | 1000 |

**[0050]** The emulsifier is dissolved in the oil fraction. The peptides and carbohydrates are dissolved in part of the water of 70°C. Minerals are dissolved separately. The oil mixture in then added to the peptide/carbohydrate solution and mixed using a high shear mixer for 3 minutes.
The pre-emulsion is then homogenised twice at 250 bars. The formula can either be pasteurised by heating at 80°C for 15 minutes and spray dried (powdered formula), or sterilised in bottles at 120°C for 10 minutes (liquid formula).

Example 8

**[0051]** The peptides can be incorporated in an instant drink mix. The recipe contains:

| | |
|---|---|
| Cys and Gly rich peptides | 15.00% |
| Whey protein concentrate 80 (Esprion 580; DMV International) | 65.00% |
| Glutamine Peptides (WGE80GPU; DMV International) | 10.00% |
| Vitamin mix (Roche) | 4.90% |
| Cocoa powder (D-11-S, ADM Cocoa, The Netherlands) | 3.00% |
| Flavour; Vanilla JSH00712F, McCormick&Co. | 1.15% |
| Flavour; Chocolate fudge FF22034, McCormick&Co. | 0.95% |
| Sweetener (Aspartame, Nutrasweet) | 0.20% |
| Total | 100% |

**[0052]** The dry ingredient are mixed and then added to 118 ml water. The solution is mixed so that the components dissolve. One serving contains 35 g of powder mix supplying approximately 500mg Cysteine and 150mg Glycine.

## Claims

1. Method for the preparation of a mixture of peptides having a cysteine content between 7-20 w/w % from a protein source, comprising cysteine containing proteins, comprising the steps of:

   a) cleaving the proteins of the protein source into peptides;
   b) digesting the peptides obtained in step a) by at least one exopeptidase, the action of which is at least attenuated at the position of a cysteine in the peptide, therewith forming digested peptides having a terminal cysteine;
   c) purifying the digested peptides.

2. Method for the preparation of a mixture of peptides having a total cysteine and glycine content of 7-20 w/w % from a protein source, comprising cysteine and glycine containing proteins, comprising the steps of:

   a) cleaving the proteins into peptides;
   b) digesting the peptides obtained in step a) by at least one exopeptidase, the action of which is at least attenuated at the position of cysteines and glycines in de peptide, therewith forming digested peptides having a terminal cysteine or glycine;
   c) purifying the digested peptides.

3. Method according to claim 1 or 2, wherein steps a) and b) are carried out simultaneously.

4. Method according to any of the preceding claims, wherein the at least one exopeptidase comprises carboxypeptidase Y.

5. Method according to any of the preceding claims, wherein the protein source comprises at least two different cysteine-containing proteins.

6. Method according to any of the preceding claims, wherein the protein source comprises at least two different proteins, at least one of which contains cysteine residues and at least one of which contains glycine residues.

7. Method according to any of the preceding claims, wherein the protein source consists of edible proteins.

8. Method according to any of the preceding claims, wherein the protein source comprises whey protein isolate and/or whey protein concentrate.

9. Method according to any of the preceding claims, wherein the protein source comprises one or more of the group, consisting of albumin, especially αlactalbumin, bovine serum albumin, wheat gluten, maize protein isolate, egg proteins, especially ovalbumin, cystatin.

10. Method according to any of the preceding claims, wherein step a) and step b) are done at conditions, wherein sulfur bridges between cysteine residues as present in the proteins of the protein source are kept intact.

11. Method according to claim 10, wherein the steps a) and b) are done at a pH of 2-8.

12. Method according to any of the preceding claims, wherein step a) comprises cleavage of the proteins by an enzyme with endopeptidase function.

13. Method according to claim 12, wherein the enzyme with endopeptidase function also has exopeptidase function, the exopeptidase function of which is attenuated at the position of cysteine.

14. Method according to claim 12 or 13, wherein the exopeptidase function of the enzyme is attenuated at the position of both glycine and cysteine.

15. Method according to claim 13 or 14, wherein the enzyme is chosen from Flavourzyme, Acid Protease A, Protease M, Protease 2A, Protease B, Corolase PN-L, Acid Protease, or a combination of one or more thereof.

16. Preparation comprising cysteine-rich peptides, comprising 7-20 w/w % cysteine.

17. Preparation comprising cysteine- and glycine-rich peptides, comprising 7-20 w/w % cysteine and glycine.

18. Preparation according to claim 17 or 18, of which at least 80% of the peptides comprise terminal cysteines and/or glycines.

19. Use of a preparation according to any of the claims 17-18 as active compound in a medicament.

20. Use of a preparation according to any of the claims 17-18 as active compound in a medicament for the treatment of conditions mediated by oxidative damage.

21. Use of a preparation according to any of the claims 17-18 as active compound in a medicament for the elevation of cellular glutathion levels in the human or animal body.

22. Use of a preparation according to any of the claims 17-18 in infant formula.

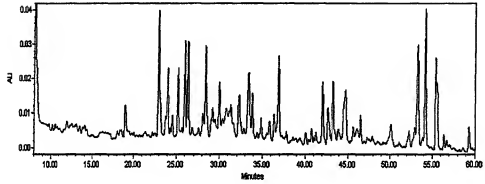
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Fig 1a

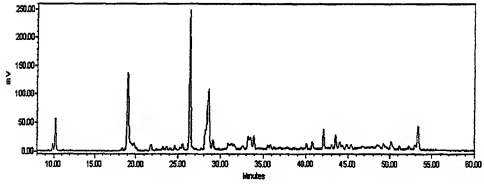
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Fig 1b

European Patent Office

# EUROPEAN SEARCH REPORT

Application Number
EP 00 20 3699

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| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int.Cl.7) |
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| A | WO 98 44807 A (BRINGE NEAL A ;MONSANTO INC (US)) 15 October 1998 (1998-10-15) * claims 1,2,4,14-17,54-61; tables 9,11 * | 1-22 | |
| A | US 5 451 412 A (GOLD PHIL ET AL) 19 September 1995 (1995-09-19) * column 6, line 18 - line 27; tables 7,9 * | 1-22 | |

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

A23J
C07K
A61K
C12P
A23L

The present search report has been drawn up for all claims

| Place of search | Date of completion of the search | Examiner |
|---|---|---|
| THE HAGUE | 4 April 2001 | Heezius, A |

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

## ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 00 20 3699

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

04-04-2001

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## (54) Cysteine/glycine rich peptides

Peptiden reich an Cystein und/oder Glycin

Peptides riches en cystéine et/ou glycine

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Remarks:

The file contains technical information submitted after the application was filed and not included in this specification



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## Description

**[0001]** The invention relates to a method for the preparation of a mixture of peptides having a cysteine- or cysteine/glycine content between 7-20 w/w %, to preparations comprising said peptides and to the use of such preparations as active compound in a medicament.

**[0002]** Peptides are defined as amino acid chains, derived from a protein; the molecular weight of the peptides is preferably between 200D and 8000D, more preferably between 1000D and 5000D.

**[0003]** In the art, there is a great demand for cysteine and cysteine/glycine comprising compounds for effective administration of said amino acids to the human or animal body. The availability of especially cysteine and to a lesser extent glycine, is a limiting factor in the syntheses of glutathion. Proper administration of cysteine, but also of glycine is therefore demanded in cases where an elevation of cellular glutathion levels in the human or animal body are needed.

**[0004]** Glutathion (GSH) is a tripeptide-thiol (L-γ-glutamyl-L-cysteinylglycine) having a broad range of vital functions, including protection of cells against oxygen intermediates, free radicals, byproducts of the oxygen requiring metabolism, and detoxification of xenobiotics. Further, glutathion seems to play a role in the prevention of cataract and oxidative DNA injury. Glutathion is therefore regarded as an important compound against oxidative stress related diseases like myocardial ischemia, cancer and cataract.

**[0005]** In view of the crucial role played by glutathion either in combating the assaults of free radical injuries or in detoxification of xenobiotics, including drug metabolites (such as cyclophosphamide, paraquat and acetaminophen) and in preventing peroxidation of cell components, a method for maintaining hepatic stores of glutathion, particularly during times of stress to the body, including chemotherapy, is needed.

**[0006]** In the art, various methods are known to increase cellular levels of glutathion. Administration to animals of the glutathion amino acid precursors glutamic acid, cysteine and glycine, may produce an increase in cellular glutathion, but there is a limit to the effectiveness of this procedure.

**[0007]** Cellular concentrations of GSH are dependent on the supply of cysteine, which is often the limiting amino acid, and which is derived from dietary protein and also by trans-sulfuration from methionine in the liver. However, administration of cysteine as free amino acid is not an ideal way to increase GSH concentrations because cysteine is rapidly metabolised and furthermore, appears to be toxic to cells at higher concentrations. Administration to animals of compounds that are transported into cells and converted intracellularly into cysteine is sometimes useful in increasing cellular glutathion levels.

**[0008]** Another way in which tissue GSH concentration may be increased is by administration of gamma glutamyl-cysteine or of gamma-glutamylcystine. The administered gamma-glutamyl amino acid is transported intact and serves as a substrate of GSH synthetase. It is also known that administration of N-acetyl-L-cysteine can often increase tissue concentrations of GSH. Other reports on using N-mercaptopropionyl glycine for increasing intracellular glutation are known. A few clinical trials have been done using mercaptopropionyl glycine to elevate intracellular glutathion.

**[0009]** That the administration of glutathion itself might lead to increased glutathion levels has also been considered. However, there is no published evidence that shows that intact glutathion enters cells. In fact, there are several reports on particular biological systems indicating that glutathion itself is not transported into cells. The increase in cellular glutathion sometimes found after administration of glutathion is due to (a) extracellular breakdown of glutathion, (b) transport into cells of free amino acids or dipeptides derived from glutathion extracellularly, and (c) intracellular resynthesis of glutathion.

**[0010]** Apart from these conventional methods for increasing glutathion levels, there have been several attempts to demonstrate how glutathion can be enhanced intracellularly. All these relate to synthetic derivatives or about intact undenatured proteins which are heat labile and none whatsoever to natural derived peptide mixtures. Some of the relevant ones are summarised below:

US Patent No 5,869,456 relates to preparation of pure alkyl esters of glutathion (95% pure) and a method for increasing intracellular glutathion levels by administering such alkyl diester of glutathion.

US patent 5,464,825 describes the method for preparation and use of N-acyl glutathion monoalkyl esters to provide increased intracellular levels of glutathion or glutathion equivalents, e.g. N-acyl glutathion or glutathion monoalkyl esters.

US patent 5,248,697 describes a method for maintaining and/or enhancing tissue or plasma levels of glutathion. The patent teaches the art of treatment of a mammal with a supranormal amount of glutamine, or a glutamine equivalent, to prevent the reduction in tissue glutathion levels associated with exposure of the mammal to a compound capable of oxidative injury to the tissue.

US patent 4,665,082 discusses the role of L-2-oxothiazolidine-4-carboxylate, a sulfur analog of 5-oxoproline, cleaved by the enzyme-5-oxo-L-prolinase to form cysteine, thus providing the basis for a cysteine delivery system by the addition of L-2-oxothiazolidine-4-carboxylate to base amino acid solutions or by injecting it directly into in vivo cells.

WO 98/44807 discloses soy protein isolates which are enriched in beta-conglycinin as to increase cysteine and methionine levels up to about 2.4 w/w %, as well as a method for producing the same.

DE patent No 4,329,857 teaches the use of thiol compounds (cysteine and its derivatives or analogues like N-acetyl cysteine, homocysteine, glutathion, 2-oxothiazolidine-4-carboxylic acid) as an agent for strengthening the immune system and immune reactions.

**[0011]** According to the present invention, a novel method for the preparation of a mixture of peptides having a cysteine content between 7-20 w/w% from a protein source, comprising cysteine containing proteins is provided. The protein source is preferably a natural protein source. The peptide mixture prepared according to this embodiment of the present invention has the advantage that it is derived from natural protein sources and will not show any adverse side-effects, whereas chemically produced cysteine derivatives as mentioned in the prior art, have shown adverse side effects. There has been found that such a preparation of a peptide mixture can be very advantageously used as cysteine source in diet supplements or in medicaments, as will be explained below.

**[0012]** The method is characterized in that it comprises the steps of:

a) cleaving the proteins of the protein source into peptides;
b) digesting the peptides obtained in step a) by at least one exopeptidase, the action of which is at least attenuated at the position of a cysteine in the peptide, therewith forming digested peptides having a terminal cysteine;
c) purifying the digested peptides.

**[0013]** In the first step a) proteins of the protein source are cleaved into smaller peptides. This cleavage can be performed by cleavage reactions, known in the art; preferably, the cleavage is performed by enzymatic hydrolysis of the peptide bonds of the protein by e.g. an endopeptidase, resulting in the peptides of about the desired length, and therewith increasing the amount of substrate for the exopeptidase. In a second step, the peptides as obtained by the cleavage reaction, are digested by at least one exopeptidase. With "at least one exopeptidase" is meant that the digestion reaction can be carried out by one or more different exopeptidases. Exopeptidases release amino acids from the terminal ends of the peptides one by one. The exopeptidase and the digestion reaction conditions are chosen such, that the exopeptidase action is at least attenuated at the position of a cysteine in the peptide. With "at least attenuated" is meant that the exopeptidase does not remove the cysteine from the peptide at the chosen reaction conditions or has very low preference for the cleavage of cysteine, therewith rendering said cleavage reaction very slow compared to cleavage of other amino acids from the peptide. By the use of such an exopeptidase and condition, the peptides are generated of which the terminal amino acids have been removed up to the cysteine residue most close to said terminus. The skilled person will be able to find conditions at which commercially available enzymes with exopeptidase function having attenuated action at the cysteine. It is to be understood that the peptides may have one or more amino acid chains that are coupled to each other by disulfide bridges of cysteine residues, present in the said amino acid chains. "A digested peptide having a terminal cysteine" therefore reflects to the fact that at least one of the termini of such a multi-chain peptide has a terminal cysteine. Of course, such a peptide may contain more than one terminal cysteine. Preferably, the enzymatic activity is inactivated before the purification step, e.g. by a pH shift or a thermal heat inactivation treatment.

**[0014]** Preferably, the exopeptidase comprises Carboxypeptidase Y (E.C.3.4.16.1.), as it has been found that this enzyme can be very effectively attenuated at cysteine residues, therewith producing peptides with terminal cysteine residues.

**[0015]** The cleavage step a) and the digestion step b) can be conducted simultaneously, e.g. by using an endopeptidase and an exopeptidase that both function at the same reaction conditions. Also, enzyme preparations can be used that have both endopeptidase and exopeptidase activity.

**[0016]** Finally, these digested peptides are purified. Suitable methods to purify the digested peptides from free amino acids, released by the exopeptidase, are known in the art. Since a difference in molecular weight is created between the cystine and glycine containing peptides and the other free amino acids, the cystine and glycine peptides can be purified using this difference. Several techniques, known in the art, could be used. Preferably the free amino acids are separated using a membrane process, preferably ultra or nanofiltration. The purification step can also advantageously comprise the use of an immobilized metal affinity chromatography step (IMAC) accordingly to Kronina et al., Journal of Chromatography A, 852 (1999) pp 261-272. The cysteine and glycine rich peptides can hereafter be dried.

**[0017]** In a special embodiment, the exopeptidase in step b) and the cleavage reaction are chosen such, that the exopeptidase is at least attenuated both at the position of a cysteine as well as of glycine in the peptide. This will result in digested peptides having predominantly a terminal cysteine or glycine.

**[0018]** The purified peptides, either enriched in cysteine residues or enriched in both cysteine and glycine residues, have shown to be very suitable sources for these limiting amino acids to be readily administered, in order to elevate the cysteine and glycine rates in the human or animal body, and may therefore elevate the intracellular glutathion levels.

**[0019]** The protein source may be any source as long as it comprises cysteine-containing proteins. In case a cysteine and glycine rich peptide preparation is to be produced, the protein source should contain proteins that contain glycine and cysteine.

**[0020]** Preferably, the protein source comprises at least two different proteins, that both contribute to the glycine and/or cysteine content of the peptides. One of the proteins may be glycine rich, whereas the second protein may be cysteine rich. The protein source can also be prepared before being subjected to the method of the present invention, by e.g. two or more protein sources before or during the cleavage step.

**[0021]** Preferably, the protein source consists of edible proteins, so that the digested peptides can be used as food additive. In a very special embodiment, the protein source comprises whey protein isolates (WPI) and/or whey protein concentrates (WPC). The terms "whey protein isolates" and "whey protein concentrates" are known in the field. Whey protein concentrate is a whey protein product having 35-80 w/w% protein, whereas whey protein isolate has a protein content of 90 w/w% or higher. An example of WPC is Esprion 580 from DMV International; an example of WPI is Bipro from Bio-isolates Ltd. Whey protein is an important cysteine source and it is thought that whey protein concentrate induces glutathion production in animal organs, see e.g. US-5 451 412. However, whey protein concentrates as such are not as suitable for the elevation of the intracellular glutathion levels compared to the peptides according to the present invention. The concentration of cystein and glycine in the intact whey proteins is much lower than in the peptides of the invention, and therefore requires much higher doses of the intact whey protein to reach an acceptable level of cystein in the application.

**[0022]** A further disadvantage of US 5 451 412 is that the use of totally undenatured whey protein products can be very costly since it requires very delicate process conditions. Whey protein isolate comprises very suitable cysteine and glycine rich proteins, such as albumin, especially α-lactalbumin and bovine serum albumin. Said proteins are advantageously used in or as starting protein source of the method according to the invention.

**[0023]** In another preferred embodiment, the protein source comprises one or more of the group consisting of albumin, especially α-lactalbumin, bovine serum albumin, egg proteins (e.g. ovalbumin, cystatin) wheat gluten, maize protein isolate.

**[0024]** Preferably, steps a) and b) are done at conditions, wherein sulfur bridges between cysteine residues as present in the proteins in the protein source are kept in the oxidised form as much as possible. In this way, cysteine-rich peptide mixtures are obtained, in which most of the cysteine residues are oxidised and coupled to other peptides through disulfide bridges. Although the correct nomenclature for cysteines in oxidized form (i.e. being coupled to another cysteine residue by a sulfur bridge) is "cystine", in this application "cysteine" is defined both as cysteine in the reduced form (having free SH-groups) as in the oxidized (cystine) form. Peptides, wherein the sulfur bridges between the cysteine residues are intact, may mimic parts of the native original protein from which the peptides are derived, therewith possibly conferring an improved biologic action compared to that of the separate peptides in reduced form. Further, the oxidized form is less reactive and therefore more stable in applications that undergo a heat treatment like pasteurization or sterilization.

**[0025]** A further advantage is the fact that many enzymes having exopeptidase activity do not cleave oxidized cysteines, whereas cysteines in reduced form may be cleaved by said enzymes from the peptides, although with a relative low activity. In order to produce peptide mixtures in native, i.e. undenatured form, steps a) and b) are preferably done at a pH between 2 and 8.

**[0026]** It is preferred to carry out the hydrolytic processes in acidic environments. At acid pH the disulfide bridges in cystine are more stable than at basic pH. [Creighton, T.E., 1993, Proteins: structures and Molecular Properties. 2nd Ed.; Freeman and Company, New York)

**[0027]** It is preferred to cleave the proteins of the protein source in step a) by an enzyme with endopeptidase function. Using such an enzyme makes it possible to cleave the proteins under undenaturing (i.e. native) conditions, resulting in undenatured cleavage products. Physical or chemical cleavage mostly implicates application of denaturing conditions that can not be used if intact native peptide mixtures are to be obtained. For this, the expopeptidase digestion should also preferably take place at undenaturing conditions. The skilled person will know the proper conditions to yield intact native peptide mixtures. "Intact native peptide" is in this content to be understood as a peptide, having the same conformation as the said peptide has in the native, functional protein.

**[0028]** In a very attractive embodiment, the enzyme with endopeptidase function also has exopeptidase function, the exopeptidase function of which is attenuated at the position of cysteine or both at glycine and cysteine. Such enzymes are known in the art and the advantage thereof is that steps a) and b) can be done simultaneously. Examples of preferred enzymes having both endopeptidase as exopeptidase functions are Flavourzyme, Acid Protease A, Protease M, Protease 2A, Protease B, Corolase PN-L, Acid Protease or a combination of one or more thereof.

**[0029]** The invention further relates to preparations comprising cysteine-rich peptides, comprising 7-20 w/w % cysteine and to such a preparation comprising 7-20 w/w % of cysteine/glycine. As indicated above, said preparations can advantageously be used for administration to animals or humans in order to effectively improve the cysteine uptake of cysteine or a combination of cysteine and glycine for e.g. elevation of the intracellular glutathion level. Preferably, at least 80% of the peptides of the preparation comprises terminal cysteines and/or glycines, which are then readily available for the human or animal body. These terminal cysteines and/or glycines are obtained by the use of the exopeptidase as discussed above.

**[0030]** Further, the invention relates to the use of a preparation according to the invention as active compound in a

medicament, especially in a medicament for treatment of conditions mediated by oxidative damage and in a medicament for the elevation of cellular glutathion levels in the human or animal body. For this, the preparation can be combined with any suitable carrier, diluent adjuvant etc. In order to obtain the medicament in the desired administration form. The preparation can also advantageously be used in an infant formula, e.g. in a breast milk substitute.

**[0031]** The invention is now illustrated in the following examples and figures which are meant to be illustrative only and not to limit the scope of the invention.

Fig. 1a shows an absorption spectrum at 214 nm of a hydrolysate according to the invention,

Fig. 1b shows a fluorescence spectrum with an excitation wavelength of 386 nm and an emission wavelength of 514 nm of the hydrolysate of fig. 1a.

Example 1

**[0032]** A 10% whey protein isolate (WPI) solution is prepared and then hydrolysed using enzymes. Several combinations of enzymes were used (Table 1).

Table 1: Enzyme(s) used

| Exp No | Enzyme 1 | Enzyme 2 |
|---|---|---|
| A | Pepsin (Merck) 0.5% | Protease M (Amano) 0.5% |
| B | Pepsin (Merck) 0.75% | Corolase LAP (Rohm) 2% |
| C | Pepsin (Merck) 0.5% | Acid Protease (EDC) 0.5% |
| D | Flavourzyme (Novo) 1% | |
| E | Acid Protease (EDC) 1% | |

Solutions 1 - 3 were first hydrolysed with pepsin for 6 hours at pH 2.0. Hereafter, the pH was increased to 7.0 using sodium hydroxide. The second enzyme was added and solutions incubated for 20 hours. The solutions containing a single enzyme were hydrolysed for 20 hours at 50°C at pH 7 and 3 for respectively Flavourzyme and Acid Protease. Hydrolytic reaction was stopped by heating the solutions to 85°C for 15 minutes. Hereafter, the free amino acids were removed from the peptides containing cysteine using ultrafiltration. A membrane with a nominal molecular weight (NMW) cut off of 1000 dalton was used. The solutions were ultrafiltered to 500% diafiltration. Protein was measured using the Kjeldahl method. Cysteine concentration was measured using the Ellmann's reagents. [Beveridge et al (1974) Journal of Food Science Volume 39, p. 49 - 51] The peptides were then freeze dried.

**[0033]** The table below lists the concentrations of both cystine and glycine in the whey protein isolate and peptides.

| | Total Cystine on protein | Total Glycine on protein |
|---|---|---|
| WPI | 3.3% | 1.9% |
| 1 | 8.5% | 2.4% |
| 2 | 11.9% | 2.7% |
| 3 | 12.4% | 2.7% |
| 4 | 9.7% | 2.1% |
| 5 | 10.9% | 3.0% . |

Example 2

**[0034]** A 10% whey protein concentrate containing 80% protein solution is prepared and then hydrolysed using 1% Acid Protease form Enzyme Development Corporation. The solution was hydrolysed for 20 hours at pH 3.0. The reaction was stopped by heating the solution to 90°C for 10 minutes. Hereafter, the solution was ultrafiltered using a membrane having a NMW cut off of 1000 dalton. Cysteine concentration was measured as a function of the %-diafiltration (table 2).

Table 2: Cysteine concentration as a function of the %-diafiltration

| | weight (g) | Protein (%) | Cysteine (%) |
|---|---|---|---|
| Hydrolysate | 172 | 71.5 | 2.1 |

Table continued

| | weight (g) | Protein (%) | Cysteine (%) |
|---|---|---|---|
| 100 % diafiltered permeate | 167 | 68.0 | 0.2 |
| 200% diafiltered permeate | 150 | 71.3 | 0.1 |
| Retentate after diafiltration | 75.0 | 71.4 | 4.1 |

Example 3

**[0035]** 100 lt of a 5% whey protein isolate solution is prepared and then hydrolysed using 2% Acid Protease form Enzyme Development Corporation. The solution was hydrolysed for 12 hours at pH 3.0. The reaction was stopped by heating the solution to 80°C for 30 minutes. Hereafter, the solution was ultrafiltered on a pilot UF unit using Koch HFK 328 membrane having a NMW cut off of 5000 dalton. The hydrolysate was split in two parts one part was filtered at the pH as is (3.8). The pH of the other part was first raised to 7.0 using sodium hydroxide after which it was ultrafiltered.

**[0036]** Cysteine concentration was measured as a function of the pH during ultrafiltration (table 3).

Table 3: Cysteine concentration as a function of the pH during ultrafiltration

| Sample | Cysteine on dry matter |
|---|---|
| Hydrolysate | 3.14 |
| Retentate UF pH 3.9 | 7.73 |
| Retentate UF pH 7.0 | 8.14 |

Example 4

**[0037]** The hydrolysate as in example 5 was nanofiltered using the Celgard NF-PES-10 membrane having a NMW cut off of dalton.
The NF-conditions were:

Pressure : 30 bar

Temperature : 50-55°C

Initial flux 58 ltrs/$m^2$/hr

End flux : 23 ltrs/$m^2$/hr

Process : concentrated to 30 ltrs and then 200% diafiltration

**[0038]** The resulting peptides in the retentate contained 12.9% Cysteine and 3.1% Glycine.

Example 5 HPLC specific for Cys Peptides

**[0039]** A Reversed Phase HPLC-method (RPC) was set-up to identify and quantitate cysteine containing peptides in a mixture of peptides. The cysteine residues were first labelled with a fluorescent label (SBD-F; 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid; Sigma F-4383). This label specifically binds to cysteine residues.
In total 300 µl sample (100µg protein per ml), 600 µl incubationbuffer (250mM borate buffer, pH 8, 5mM EDTA), 300 µl fluorescent probe (0,1 % (W/V) in water), 297 µl $H_2O$ and 3 µl TBP (tributylphosphine, Fluka) are pipetted in a vial. The vial is capped, the mixture mixed well and incubated at 60C for 10 minutes. The final concentration of the sample is 0.02 mg/ml.
Hereafter the mixture is cooled to room temperature by putting on ice. Solution is filtered using 0.45µm PVDF filter (millipore, Millex-HV).
The filtered solutions are analysed by reversed phase chromatography using a Widepore C18 5µm RPC column (Baker). The binding buffer consisted of demineralised water/0.1 % TFA (trifluoroacetic acid) and the peptides were eluted using a acetonitril/0.083% TFA buffer (buffer B). The level of Buffer B was increased to 60% in 90 minutes, whereafter tightly

bound material was removed by running 100% buffer for 20 minutes. The injection volume was 150µl sample. The peptides are detected by measuring absorption at 214nm and fluorescence (excitation and emission wavelengths respectively 386nm and 514nm), see fig.1.

**[0040]** Upper panel of fig.1 shows the hydrolysate from example 1 before separation detecting the peptides by measuring adsorption at 214nm Lower panel shows the same hydrolysate detecting specific cysteine containing peptides measuring the fluorescence.

Example 6

**[0041]** The cysteine and glycine rich peptides can be used in clinical enteral nutrition formulas. A recipe for such formula is as follows:

| | |
|---|---|
| Cys and Gly rich peptides | 5.00% |
| Calcium Caseinate (DMV International) | 1.96% |
| Malto dextrin DE-20 | 14.0% |
| Emulsifyer (Stemphil E60; Stem) | 0.30% |
| Oil-mix (50% sunflower; 20% MCT; 30% soy-oil) | 4.90% |
| Sodium Chloride | 0.09% |
| Tri-Calcium Phosphate | 0.95% |
| Magnesium Chloride | 0.15% |
| Calcium di-Hydrogen Phosphate | 0.13% |
| Tri-Sodium Citrate | 0.086% |
| Water | 77.99% |
| Total | 100% |

**[0042]** Caseinate is dissolved in part of the demineralised water at 60°C; emulsifier is dissolved in the oil-mix; salts are dissolved in 75 ml water. Hereafter, the oil mix, salt, malto dextrin solution and residual water are subsequently mixed in the caseinate solution. This mixture is homogenised twice at 350 bar and at 70°C. The Cysteine & Glycine rich peptides are then dissolved in the emulsion. The pH is adjusted to 7.0 - 7.1 using sodium hydroxyde and then the product is retort sterilised for 10 minutes at 121°C.

Example 7

**[0043]** The peptides can be used in infant formula's. A model recipe is as follows:

| Component | Concentration (g/lt) |
|---|---|
| Cys and Gly rich peptides | 10.0 |
| WE80BG (whey protein hydrolysate DMV International) | 10.0 |
| Edible Lactose (DMV International) | 30.0 |
| Malto dextrin DE-20 | 23.0 |
| Corn Syrup Solids | 25.0 |
| Emulsifier (Stemphil E60; Stera) | 5.0 |
| Oil-mix (45% sunflower; 25% MCT; 30% soy-oil) | 40.0 |
| Calcium ortho phosphate | 1.8 |
| Calcium carbonate | 1.3 |
| Magnesium Chloride | 0.3 |
| Potassium Chloride | 0.4 |
| Tri-Sodium Citrate | 0.5 |
| Water | 852.7 |
| Total | 1000 |

**[0044]** The emulsifier is dissolved in the oil fraction. The peptides and carbohydrates are dissolved in part of the water of 70°C. Minerals are dissolved separately. The oil mixture in then added to the peptide/carbohydrate solution and mixed

using a high shear mixer for 3 minutes.
The pre-emulsion is then homogenised twice at 250 bars. The formula can either be pasteurised by heating at 80°C for 15 minutes and spray dried (powdered formula), or sterilised in bottles at 120°C for 10 minutes (liquid formula).

Example 8

**[0045]** The peptides can be incorporated in an instant drink mix. The recipe contains:

| | |
|---|---|
| Cys and Gly rich peptides | 15.00% |
| Whey protein concentrate 80 (Esprion 580; DMV International) | 65.00% |
| Glutamine Peptides (WGE80GPU; DMV International) | 10.00% |
| Vitamin mix (Roche) | 4.90% |
| Cocoa powder (D-11-S, ADM Cocoa, The Netherlands) | 3.00% |
| Flavour; Vanilla JSH00712F, McCormick&Co. | 1.15% |
| Flavour; Chocolate fudge FF22034, McCormick&Co. | 0.95% |
| Sweetener (Aspartame, Nutrasweet) | 0.20% |
| Total | 100% |

**[0046]** The dry ingredient are mixed and then added to 118 ml water. The solution is mixed so that the components dissolve. One serving contains 35 g of powder mix supplying approximately 500mg Cysteine and 150mg Glycine.

## Claims

1. Method for the preparation of a mixture of peptides having a total cysteine and glycine content of 7-20 w/w % from a protein source comprising cysteine and glycine containing proteins, comprising the steps of:

   a) cleaving the proteins into peptides;
   b) digesting the peptides obtained in step a) by at least one exopeptidase, the action of which is at least attenuated at the position of cysteines and glycines in the peptide, therewith forming digested peptides having a terminal cysteine or glycine;
   c) purifying the digested peptides.

2. Method according to claim 1, for preparin a mixture of peptides having a cysteine content of 7-20 w/w %.

3. Method according to claim 1 or 2, wherein steps a) and b) are carried out simultaneously.

4. Method according to any of the preceding claims, wherein the at least one exopeptidase comprises carboxypeptidase Y.

5. Method according to any of the preceding claims, wherein the protein source comprises at least two different cysteine-containing proteins.

6. Method according to any of the preceding claims, wherein the protein source comprises at least two different proteins, at least one of which contains cysteine residues and at least one of which contains glycine residues.

7. Method according to any of the preceding claims, wherein the protein source consists of edible proteins.

8. Method according to any of the preceding claims, wherein the protein source comprises whey protein isolate and/or whey protein concentrate.

9. Method according to any of the preceding claims, wherein the protein source comprises one or more of the group, consisting of albumin, especially α-lactalbumin, bovine serum albumin, wheat gluten, maize protein isolate, egg proteins, especially ovalbumin, cystatin.

10. Method according to any of the preceding claims, wherein step a) and step b) are done at conditions, wherein sulfur

bridges between cysteine residues as present in the proteins of the protein source are kept intact.

11. Method according to claim 10, wherein the steps a) and b) are done at a pH of 2-8.

12. Method according to any of the preceding claims, wherein step a) comprises cleavage of the proteins by an enzyme with endopeptidase function.

13. Method according to claim 12, wherein the enzyme with endopeptidase function also has exopeptidase function, the exopeptidase function of which is attenuated at the position of cysteine.

14. Method according to claim 12 or 13, wherein the exopeptidase function of the enzyme is attenuated at the position of both glycine and cysteine.

15. Method according to claim 13 or 14, wherein the enzyme is chosen from Flavourzyme, Acid Protease A, Protease M, Protease 2A, Protease B, Corolase PN-L, Acid Protease, or a combination of one or more thereof.

16. Method for the preparation of a mixture of peptides having a cysteine content of 7-20 w/w % from a protein source comprising cysteine-containing proteins, comprising the steps of:

a) cleaving the proteins of the protein source into peptides;
b) subsequently or simultaneously digesting the peptides obtained in step a) by at least one enzyme having endopeptidase and exopeptidase functions chosen from Flavourzyme, Acid Protease A, Protease M, Protease 2A, Protease B, Corolase PN-L and Acid Protease, therewith forming digested peptides having a terminal cysteine;
c) purifying the digested peptides.

17. Method according to any of the preceding claims, wherein the action of the exopeptidase is at least attenuated at the position of cysteines and glycines in the peptide.

18. Preparation comprising a mixture of cysteine- and glycine-rich peptides, comprising 7-20 w/w % cysteine and glycine.

19. Preparation according to claim 18, the mixture of cysteine- and glycine-rich peptides comprising 7-20 w/w % cysteine.

20. Preparation according to claim 18 or 19, of which at least 80% of the peptides comprise terminal cysteines and/or glycines.

21. Use of a preparation according to any of the claims 18-20 as active compound in a medicament.

22. Use of a preparation according to any of the claims 18-20 as active compound in a medicament for the treatment of conditions mediated by oxidative damage.

23. Use of a preparation according to any of the claims 18-20 as active compound in a medicament for the elevation of cellular glutathione levels in the human or animal body.

24. Use of a preparation according to any of the claims 18-20 in infant formula.

**Patentansprüche**

1. Verfahren zur Herstellung einer Mischung aus Peptiden mit einem Gesamtcystein-und -glycingehalt von 7 bis 20 G/G% aus einer Proteinquelle, umfassend cystein-und glycinhaltige Proteine, umfassend die folgenden Schritte:

a) Spaltung der Proteine in Peptide;
b) Verdau der im Schritt a) erhaltenen Peptide durch mindestens eine Exopeptidase, deren Wirkung an der Stellung von Cysteinen und Glycinen in dem Peptid zumindest abgeschwächt ist, dadurch Bildung verdauter Peptide mit einem terminalen Cystein oder Glycin;
c) Reinigung der verdauten Peptide.

2. Verfahren gemäß Anspruch 1, zur Herstellung einer Mischung aus Peptiden mit einem Cysteingehalt von 7 bis 20 G/G%.

3. Verfahren gemäß Anspruch 1 oder 2, wobei die Schritte a) und b) gleichzeitig durchgeführt werden,

4. Verfahren gemäß einem der vorstehenden Ansprüche, wobei die mindestens eine Exopeptidase Carboxypeptidase Y umfasst.

5. Verfahren gemäß einem der vorstehenden Ansprüche, wobei die Proteinquelle mindestens zwei unterschiedliche cysteinhaltige Proteine umfasst

6. Verfahren gemäß einem der vorstehenden Ansprüche, wobei die Proteinquelle mindestens zwei unterschiedliche Proteine umfasst, wobei mindestens eins davon Cysteinreste und mindestens eins Glycinreste enthält.

7. Verfahren gemäß einem der vorstehenden Ansprüche, wobei die Proteinquelle aus essbaren Proteinen besteht

8. Verfahren gemäß einem der vorstehenden Ansprüche, wobei die Proteinquelle Molkeproteinisolat und/oder Molkeproteinkonzentrat umfasst

9. Verfahren gemäß einem der vorstehenden Ansprüche, wobei die Proteinquelle ein oder mehr aus der Gruppe, bestehend aus Albumin, insbesondere α-Lactalbumin, Rinderserumalbumin, Weizengluten, Maisproteinisolat, Elproteine, insbesondere Ovalbumin, Cystatin umfasst.

10. Verfahren gemäß einem der vorstehenden Ansprüche, wobei die Schritte a) und b) unter Bedingungen durchgeführt Werden, unter denen die Schwefelbrücken zwischen den Cysteinresten, wie sie in den Proteinen der Proteinquelle vorliegen, intakt bleiben.

11. Verfahren gemäß Anspruch 10, wobei die Schritte a) und b) bei einem pH von 2 bis 8 durchgeführt werden.

12. Verfahren gemäß einem der vorstehenden Ansprüche, wobei Schritt a) die Spaltung der Proteine durch ein Enzym mit Endopeptidasefunktion umfasst.

13. Verfahren gemäß Anspruch 12, wobei das Enzym mit Endopeptidasefunktion auch eine Exopeptidasefunktion aufweist, wobei die Exopeptidasefunktion an der Position des Cysteins abgeschwächt ist.

14. Verfahren gemäß Anspruch 12 oder 13, wobei die Exopeptidasefunktion des Enzyms an der Position von sowohl Glycin als auch Cystein abgeschwächt ist

15. Verfahren gemäß Anspruch 13 oder 14, wobei das Enzym gewählt ist aus Flavourzym, saure Protease A, Protease M, Protease 2A, Protease B, Corolase PN-L, saure Protease oder einer Kombination von einem oder mehreren davon.

16. Verfahren zur Herstellung einer Mischung aus Pepfiden mit einem Cysteingehalt von 7 bis 20 G/G% aus einer Proteinquelle, umfassend cysteinhaltige Proteine, umfassend die folgenden Schritte:

a) Spaltung der Proteine aus der Proteinquelle in Peptide;
b) darauffolgend oder simultan Verdau der Peptide, die in Schritt (a) erhalten wurden, durch mindestens ein Enzym mit Endopeptidase- und Exopeptidasefunktionen, gewählt aus Flavourzym, saurer Protease A, Protease M, Protease 2A, Protease B, Corolase PN-L und saurer Protease, wodurch verdaute Peptide mit einem terminalen Cystein gebildet werden;
c) Reinigung der verdauten Peptide.

17. Verfahren gemäß einem der vorstehenden Ansprüche, wobei die Wirkung der Exopeptidasen an der Stellung von Cysteinen und Glycinen in dem Peptid zumindest abgeschwächt ist.

18. Präparation, umfassend eine Mischung cystein- und glycinreicher Peptide, umfassend 7 bis 20 G/G% Cystein und Glycin.

19. Präparation gemäß Anspruch 18, wobei die Mischung cystein- und glycinreicher Peptide 7 bis 20 G/G% Cystein umfasst.

20. Preparation gemäß Anspruch 18 oder 19, wobei mindestens 80 % der Peptide terminale Cysteine und/oder Glycine umfassen.

21. Verwendung einer Präparation gemäß einem der Ansprüche 18 bis 20 als aktive Verbindung in einem Medikament

22. Verwendung einer Präparation gemäß einem der Ansprüche 18 bis 20 als aktive Verbindung in einem Medikament zur Behandlung von Zuständen, die durch einen oxidativen Schaden vermittelt worden.

23. Verwendung einer Präparation gemäß einem der Ansprüche 18 bis 20 als aktive Verbindung in einem Medikament für die Anhebung von zellulären Glutathionniveaus im menschlichen oder tierischen Körper.

24. Verwendung einer Präparation gemäß einem der Ansprüche 18 bis 20 bei Kleinfindformeln.

**Revendications**

1. Procédé pour la préparation d'un mélange de peptides ayant une teneur totale en cystéine et en glycine de 7-20% p/p issue d'une source de protéine, comprenant des protéines contenant de la cystéine et de la glycine, comprenant les étapes consistant à ;

   a) cliver les protéines en peptides ;
   b) digérer les peptides obtenus à l'étape a) avec au moins une exopeptidase, dont l'action est au moins atténuée au niveau de la position des cystéines et des glycines dans le peptide, formant ainsi des peptides digérés ayant une cystéine ou une glycine terminale;
   c) purifier les peptides digérés.

2. Procédé selon la revendication 1, pour la préparation d'un mélange de peptides ayant une teneur en cystéine de 7-20% p/p.

3. Procédé selon la revendication 1 ou la revendication 2, dans lequel les étapes a) et b) sont réalisées simultanément.

4. Procédé selon l'une quelconque des revendications précédentes, dans lequel la au moins une exopeptidase comprend la carboxypeptidase Y.

5. Procédé selon l'une quelconque des revendications précédentes, dans lequel la source de protéine comprend au moins deux protéines différentes contenant de la cystéine.

6. Procédé selon l'une quelconque des revendications précédentes, dans lequel la source de protéine comprend au moins deux protéines différentes, dont au moins une contient des résidus cystéines et dont au moins une contient des résidus glycine.

7. Procédé selon l'une quelconque des revendications précédentes, dans lequel la source de protéine est constituée de protéines comestibles.

8. Procédé selon l'une quelconque des revendications précédentes, dans lequel la source de protéine comprend un isolat de protéine de lactosérum et/ou un concentré de protéine de lactosérum.

9. Procédé selon l'une quelconque des revendications précédentes, dans lequel la source de protéine comprend une ou plusieurs des sources du groupe comprenant l'albumine, en particulier l'α-lactalbumine, l'albumine sérique bovine, la gluten de blé, un isolat de protéine de maïs, des protéines de l'oeuf, en particulier l'ovalbumine, la cystatine.

10. Procédé selon l'une quelconque des revendications précédentes, dans lequel l'étape a) et l'étape b) sont réalisées dans des conditions dans lesquelles les ponts sulfure entre les résidus cystéines présents dans les protéines de la source de protéine sont conservés intacts.

11. Procédé selon la revendication 10, dans lequel les étapes a) et b) sont réalisées à un pH de 2-8.

12. Procédé selon l'une quelconque des revendications précédentes, dans lequel l'étape a) comprend le clivage des protéines par une enzyme dotée d'une fonction d'andopeptidase.

13. Procédé selon la revendication 12, dans lequel l'enzyme dotée d'une fonction d'endopeptidase a aussi une fonction d'exopeptidase, sa fonction d'exopeptidase étant atténuée au niveau de la position de la cystéine.

14. Procédé selon la revendication 12 ou la revendication 13, dans lequel la fonction d'exopeptidase de l'enzyme est atténuée au niveau de la position de la glycine et de la cysteine.

15. Procédé selon la revendication 13 ou la revendication 14, dans lequel l'enzyme est choisie parmi Flavourzyme, la protéase acide A, la protéase M, la protéase 2A, la protéase B, Corolase PN-L, la protéase acide ou une association d'une ou plusieurs de celles-ci.

16. Procédé pour la préparation d'un mélange de peptides ayant une teneur en cystéine de 7-20% p/p issue d'une source de protéine, comprenant des protéines contenant de la cystéine, comprenant les étapes consistant à :

a) cliver les protéines de la source de protéine en peptides;
b) digérer ensuite ou simultanément les peptides obtenus à l'étape a) avec au moins une enzyme ayant des fonctions d'endopeptidase et d'exopeptidase choisie parmi flavourzyme, la protéase acide A, la protéase M, la protéase 2A, la protéase B, Corolase FN-L et la protéase acide, formant ainsi des peptides digérés ayant une cystéine terminale;
c) purifier les peptides digérés.

17. Procédé selon l'une quelconque des revendications précédentes, dans lequel l'action des exopeptidases est au moins atténuée au niveau de la position des cystéines et des glycines dans le peptide.

18. Préparation comprenant un mélange de peptides riches en cystéine et en glycine, comprenant 7-20% p/p de cystéine et de glycine.

19. Préparation selon la revendication 18, dans laquelle la mélange de peptides riches en cystéine et en glycine comprend 7-20% p/p de cystéine.

20. Préparation selon la revendication 18 ou la revendication 19, dont au moins environ 80% des peptides comprennent des cystéines et/ou des glycines terminales.

21. Utilisation d'une préparation selon l'une quelconque des revendications 18 à 20 comme composé actif dans un médicament.

22. Utilisation d'une préparation selon l'une quelconque des revendications 18. à 20 comme composé actif dans un médicament pour le traitement d'états provoqués par une lésion oxydative.

23. Utilisation d'une préparation selon l'une quelconque das revendications 18-20 comme composé actif dans un médicament pour l'élévation des concentrations de glutation cellulaire dans le corps humain ou animal.

24. Utilisation d'une préparation selon l'une quelconque des revendications 18 à 20 dans une formule pour nourrissons.

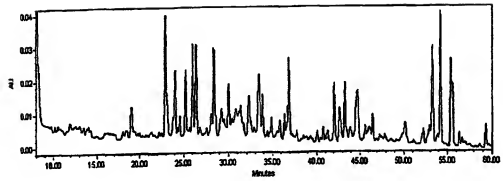
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Fig 1a

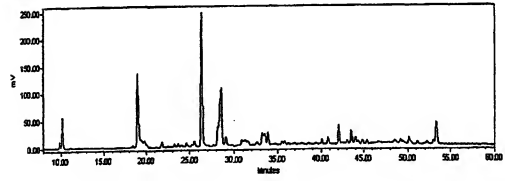
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Fig 1b

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(54) Title: APPETITE SUPPRESSION

(57) Abstract

A method and composition for reducing appetite and carbohydrate craving using precursors for the neurotransmitters serotonin, dopamine, norepinephrine and histamine, which include the precursors tryptophan, phenylalanine, tyrosine and histidine. The precursors are combined together and with xanthines for synergistic effect permitting advantageously lower doses of the precursors. Concomitant administration of histidine with any of tryptophan, phenylalanine and tyrosine produces a potentiated effect in appetite suppression. Xanthines, including theobromine, caffeine and cocoa, act as potentiators of the precursors, individually and in combinations of precursors. Separate formulations with xanthines of tyrosine and/or phenylalanine are used conjointly with a formulation of tryptophan with xanthines, each administered separately at intervals of at least 20 minutes. Hydrolyzed protein is utilized as a natural tryptophan source for the combinations, together with an insulin producing carbohydrate to remove from the blood stream other amino acids competing for transport across the blood-brain barrier. Alternatively, unhydrolyzed protein may be administered along with a proteolytic enzyme to produce tryptophan in the gastrointestinal tract.

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## Description

# APPETITE SUPPRESSION

## Technical Field

This invention relates generally to dietary supplements for reducing appetite and decreasing carbohydrate craving. There has been increasing attention to weight control since obesity is associated with an increased mortality rate, diabetes mellitus, hypertension, heart disease and stroke. The attention to reducing obesity has lead to the introduction of sugar-free and fat-free foods, diet plans, weight reduction programs, artificial fats, and pharmaceutical agents to alter both appetite and carbohydrate craving. Despite the desirability of reducing weight and the proliferation of products to aide in weight reduction, the weight of the population continues to rise. It is now estimated that more than 40% of the population is significantly overweight. At any given time approximately 25% of the population is on a diet, leading to undesirable "yo-yo" effects from repeated dieting. The failure of weight reduction products to achieve and to sustain weight loss can be attributed to several factors. These include the relative ineffectiveness of the individual approaches, side effects of weight loss products, and the cost of a sustained weight loss program. Accordingly, there is a need for an effective program based on safe naturally occurring agents. Such a program will allow weight loss with reduced side effects and reduction of costs.

## Background Art

One major component of a successful weight loss program is appetite suppression. Appetite suppression has been achieved with administration of amphetamines, antidepressants, both soluble and insoluble fibers, serotonin precursors, and prescription drugs which enhance serotonin activity. All of these techniques, as currently applied, have significant disadvantages.

Amphetamines are well known to reduce appetite. Dexedrine and related agents including ephedrine and pseudoephedrine reduce appetite. These agents either produce agitation, addiction or nerve damage (dexedrine), or produce rapid attenuation of effect (ephedrine or pseudoephedrine). Phentermine, an amphetamine-like molecule, is approved for use as an appetite suppressant, but must be administered by prescription. This results in increased costs associated with physician visits. Additionally, phentermine can only be used for short periods when administered by itself. It is believed that the amphetamines, including phentermine, suppress appetite in part through their effects on brain dopamine. Phentermine also can cause hypertension, heart irregularities and agitation. Thus, the amphetamines and related agents can be used for appetite reduction, but at substantial cost and with known, often unacceptable side effects.

One approach, introduced by Wurtman and associates in 1978, was to use precursors of brain serotonin to reduce appetite for carbohydrate. Serotonin within the hypothalamic region of the brain is known to reduce craving for carbohydrates. In Wurtman, et al U.S. Patent No. 4,210,637, a composition and method for selectively suppressing appetite for carbohydrates is described. This method includes the administration of the serotonin precursor, tryptophan, along with a carbohydrate that causes insulin secretion. Secretion of insulin moves amino acids other than tryptophan from the bloodstream into the tissues. This removes amino acids from the blood which compete with tryptophan for transport across the blood-brain barrier. This carbohydrate-initiated insulin effect on circulating amino acids maximizes delivery of tryptophan to the hypothalamus.

The dose of tryptophan proposed by Wurtman is between 10 and 100 mg per kg. in rats. For a 70 kg man, the dose would range between 700 and 7,000 mg to potentially achieve similar effects. When Wurtman applied tryptophan administration to humans in an amount of 2,300 mg per day, there was no consistent effect on appetite suppression. Moreover, the regulatory agency in the United States, the Food and Drug Administration (FDA), has found that tryptophan in doses of more than 100 mg per day may be unsafe. The FDA has determined that doses of tryptophan in excess of 100 mg per day may

potentially cause muscle damage. Accordingly, tryptophan is not being used alone, or administered with a carbohydrate, as an appetite aide.

Wurtman, et al in U.S. Patent No. 4,309,445 described a composition and method using d-fenfluramine to block intermittent carbohydrate cravings. This method disclosed that d-fenfluramine and the related isomer l-fenfluramine selectively reduces carbohydrate craving. Wurtman, et al, in U.S. Patent 4,687,763 disclosed that tryptophan can increase brain serotonin levels when given with melatonin. In this patent Wurtman, et al, disclosed that oral administration of tryptophan can increase brain serotonin and that increased brain serotonin leads to reduced carbohydrate craving. The amount of tryptophan used by Wurtman, et al, were consistently been between 2 and 100 mg/kg. of body weight per dose. These amounts are significantly above the current FDA safety guidelines of less than 1.6 mg/kg per day of supplemental tryptophan, particularly if the tryptophan comes from bacterial synthesized sources.

The FDA only allows naturally occurring protein to be used as a source of supplemental tryptophan. Both intact and "predigested" (enzyme hydrolyzed), forms of naturally occurring protein may be used. Naturally occurring protein contains approximately 1.6 % tryptophan. The amount of tryptophan in naturally occurring protein has previously been considered insufficient to produce a reduction in carbohydrate craving. This is due to the presence of other amino acids which compete for absorption with the small amount of tryptophan present in protein. In a recent FDA publication, it was concluded that there was insufficient evidence that tryptophan reduces appetite in doses considered safe. There is no known prior art suggesting the use of predigested protein as a source of tryptophan for appetite suppression.

Tyrosine is a precursor of brain dopamine. Amphetamines stimulate the release of dopamine. Brain dopamine is associated with the appetite suppressing effects of amphetamine-like agents. To date, a food supplement has not been used to enhance the release of dopamine without using amphetamines or amphetamine-like agents such as ephedrine or pseudoephedrine. Wurtman, et al, in U.S. Patent No. 4,673,689 disclose that tyrosine can be used to potentiate the sympathomimetic agents such as ephedrine or pseudoephedrine. However, this patent contains no disclosure or suggestion of any

usefulness or synergism for any purpose for combining tyrosine with any other agents active in the central nervous system.

Histidine is a precursor of histamine in the brain. It has been reported that histamine and its precursor histidine will decrease the food intake of experimental animals (rats) when administered by intraperitoneal injection ("Manipulation of Central Nervous System Histamine, Histaminergic Receptors (H1) Affects Food Intake in Rats," Mercer et al., J. of Nutrition, 1994, Vol. 24, pp 1029-1036) ) However, the effectiveness of either histamine or its precursor histidine for suppression of appetite by oral administration or at dosage levels at which the known side effects could be tolerated has not been elucidated.

Chocolate, particularly the cocoa powder, contains among other active ingredients, the xanthines theobromine and caffeine; as well as biogenic amines such as phenylethylamine. These agents influence the activity of both serotonin and dopamine. Xanthines are known to increase the release of both dopamine and serotonin. Neither chocolate or cocoa powder have been used as appetite suppressants either alone or in combination with neurotransmitter precursors such as tryptophan or tyrosine. Phenylethylamines are also known to stimulate the release of serotonin and dopamine. Phenylethylamines are also known to act as inhibitors of the enzyme monoamine oxidase (MAO), which breaks down serotonin and dopamine. Chocolate has been used both directly and indirectly, knowingly and unknowingly, as a mood elevator. The mechanism of chocolate's appeal has, heretofore, not been specifically defined. Most common knowledge attributes the appeal of chocolate to its taste, not to neurotransmitter affects.

In 1992, Wientraub observed that phentermine and fenfluramine when used together induced long term weight loss, reduced appetite and reduced carbohydrate craving. Fenfluramine is the mixture of the dextro and levo forms of fenfluramine. The results of using phentermine and fenfluramine in combination was attributed to their separate effects on serotonin and dopamine. Using this combination of prescription drugs, weight loss could be sustained for months to years. Accordingly, there has been a substantial increase in the use of the phentermine-fenfluramine approach to weight loss despite the l;ack of reuglatory approval of the the combination. Many regulatory agencies limit the use of either agent to short periods ranging from 7 days to 1

month. In addition, the use of fenfluramine has been associated with the side effect of pulmonary hypertension and heart valve disease in rare instances. The use of d-fenfluramine induces grogginess in many subjects and is expensive, often costing US$5.00 per day for the drug. This cost is in addition to multiple visits to physicians for monitoring of treatment which may last many months or years. Also, phentermine is an amphetamine-like drug whose long term effects are unknown. Accordingly, there is a need for a low cost program that emulates the effects of the phentermine-fenfluramine therapies that can be applied to a large number of individuals without repetitive physician monitoring. Ideally, the components of such a program would be formulated from low cost ingredients which are not drug.

## Disclosure of the Invention

This invention has the object of achieving appetite suppression and reduced carbohydrate craving without large doses of fibers, amphetamines, antidepressants, or other prescription drugs. This invention also has the object of enabling use of readily available, low cost, safe, plant-derived agents and to provide appetite suppression with such agents at reduced dosage to minimize the possibility of side effects.

This invention provides methods and compositions for suppressing appetite based upon the discovery that certain neurotransmitter precursors will act synergistically with each other and with certain neurotransmitter potentiators in suppressing appetite and reducing carbohydrate craving. In particular, neurotransmitter precursors for the neurotransmitters serotonin, dopamine, norepinephrine and histamine, which contain an amine group and include tryptophan, phenylalanine, tyrosine and histidine, are orally administered in reduced doses concomitantly with one or more xanthines, and particularly caffeine and/or theobromine effectively to suppress appetite. When administered alone, these neurotransmitter precursors require unacceptably high doses in order to suppress appetite.

In a further aspect of this invention histidine is administered concommitantly with either tryptophan, phenylalanine or tyrosine with synergistic effect to suppress appetite, either with or without the concomitant administration of a xanthine. Tryptophan may be

adminstered conjointly with phenylalanine or tyrosine with beneficial effect, during the same day but with administration of one seperated by at least 20 minutes of the other, to avoid competition between them for entry across the blood-brain barrier.

In another feature of the invention the neurotransmitter precursor and potentiators are administered in accordance with this invention in naturally occurring forms long considered safe for ingestion as a food stuff The neurotransmitter precursor tryptophan may be administered in the form of natural proteins which have been hydrolyzed to release amino acid residues including tryptophan. The predigested protein allows delivery of free amino acids so that a rapid effect can be produced. The hydrolyzed protein is advantageously administered concomitantly with a carbohydrate to a subject having an empty stomach (i.e. at least an hour after eating) to trigger insulin secretion to clear from the bloodstream competing amino acids that would otherwise block passage of tryptophan across the blood-brain barrier, thereby maximizing the absorption of naturally occurring tryptophan. This insulin-mediated effect on amino acids allows sufficient tryptophan to be delivered to the brain so that the desired effects are achieved

In a related embodiment, rather than administering prehydrolyzed protein, the protein source for the tryptophan may be administered in unhydrolyzed form, together with a proteolytic enzyme, so that hydrolysis occurs in the gastrointestinal tract to release the tryptophan.

Xanthines are also advantageously derived from natural sources long employed in foodstuffs, such as cocoa, tea, coffee and the like. Cocoa in particular provides a unique source of a combination of both the xanthines caffeine and theobromine and phenylethylamine that is quite palatable and considered safe.

Dosage forms are provided to advantageously and conveniently carry out the foregoing methods with reduced dosages consistent with effective suppression of appetite. The single dosage forms constitute, pills, capulets and other forms individualized for administering the appropriate single dose quantities of the selected constituents. The amount of tryptophan in the dosage forms is from about 2.5 to 100 milligrams, the amount of tyrosine is from about 10 to 700 milligrams,

the amount of histidine from about 1 to 500 milligrams. Where they present in the dosage forms, the xanthine theobromine is in the range of from about 1 mg. to 2 gm. or higher. Where cocoa is employed as the xanthine source, it may be present in the single dosage form in the amount of about 1 mg. to 2 grams or higher. Where, hydrolyzed protein is the source of tryptophan, the amount of hydrolyzed protein may be between one half of a gram. and 30 grams or higher. Desirably, the amount of hydrolyzed protein is selected to provide therein an amount of tryptophan of between 2.5 to 100 milligrams.

These combinations of agents, due to their surprising synergism, allows the dose of the individual neurotransmitter precursors to be reduced, thus reduce side effects and to reduce component doses to levels generally considered safe by regulatory agencies, such as the FDA. They additional enable the use of naturally occurring protein and plant-derived substances instead of drugs.

Under FDA regulations supplemental tryptophan cannot be synthesized by man-made processes and thus they must be derived from naturally occurring protein, either animal or vegetable. The FDA further stipulates that the dose of added tryptophan cannot exceed 100 mg per day, or 1.43 mg/kg per day. The preferred source for our invention is vegetable protein and a dose of tryptophan is 45 mg/dose or 0.71 mg/kg per day. The amount of tryptophan in the embodiment using predigested protein can be as low as 15 to 40 mg per dose. These doses of tryptophan, which comply with the FDA limitations, would be ineffective in the absence of the xanthines

**Best Mode of Carrying Out The Invention**

The following description illustrates the manner in which the principles of the invention are applied but is not to be construed as limiting the scope of the invention.

Serotonin, dopamine, norepinephrine and histamine form a class of neurotransmitters that are active in the CNS to affect appetite, either stimulating the release of corticotropin-releasing factor (CRF), which suppresses appetite, or suppressing the release and/or activity of neuropeptide Y, which stimulates appetite. Serotonin, norepinephrine

and histamine all stimulate the release of CRF. Dopamine suppresses neuropeptide Y. Histamine additionally promotes neuron firing.

The precursors for this class of neurotransmitters, all of which contain an amine group, include tryptophan for serotonin, phenylalanine and tyrosine for both dopamine and norepinephrine and histidine for histamine. In this invention, these precursors are employed in combination with each other and in combination with xanthines to potentiate the effect on appetite suppression by the respective neurotransmitters of this class.

The precursors are employed in this invention to enhance the synthesis of their respective neurotranmitters and since serotonin, phenylalanine, tyrosine and histidine all enhance synthesis of neurotransmitters that stimulate release of CRF, these precursors all thereby indirectly stimulate release of CRF. Additionally, phenylalanine and tyrosine indirectly suppresses neuropeptide Y through enhancement of the synthesis of dopamine as well. Also, histidine promotes neuron firing thereby indirectly stimulating synthesis of norepinephrine, tyrosine and serotonin.

The precursors may be employed in this invention in pure form, e.g. exogenous material synthesized or derived from animal or vegetable protein, particularly purified extracts isolated from the amino acid residues in enzyme hydrolyzed proteins. However, a source for the precursor tryptophan particularly useful in this invention, both because it is a natural food source and because of the regulatory restrictions, are proteins, either enzyme hydrolyzed prior to administration to release tryptophan or unhydrolyzed protein to be administered along with a proteolytic enzyme that will liberate the tryptophan in the gastrointestinal tract. Commercial preparations of predigested proteins, typically from milk-derived protein, such as casein or whey, are available and may be administered separately or in composition with histidine and/or a xanthine.

Where the tryptophan is to be administered in the form of a predigested protein or a protein to be enzyme hydrolyzed upon administration, it is important in this invention to administer the protein concomitantly with a carbohydrate, and particularly sugar, dextrins, starch and the like, in order to cause release of insulin to

remove from the blood stream the other amino acids competing with tryptophan for transport across the blood-brain barrier.

Where unhydrolyzed protein is administered together with a proteolytic enzyme, soluble proteins, such as albumin, are preferred, for ease of breakdown. Whey, casein and soy are convenient protein sources. Proteolytic enzymes may include papain, chymopapain, bromelin, trypsin and pepsin.

Xanthines constitute a class of non-selective adenosine antagonists and they include theobromine, caffeine and theophylline. They are capable of promoting release of the neurotransmitters serotonin, dopamine and histamine. and they potentiate neurotransmitter synthesis for each when administered in accordance with this invention. Combining xanthines, and neurotransmitter precursors allows the desired effects to be achieved with reduced, safe, doses of neurotransmitter precursors.

The xanthines may be used in the form of their free compounds or as their salts, adducts or other derivatives, for example citrated caffeine, theophylline ethylenediamine, theophylline sodium acetate, sodium glycinate, the choline salt, the theophylline derivatives theophylline-megumine and dyphylline, theobromine calcium salicylate, sodium acetate or sodium salicylate.

A particularly suitable source of xanthines for use in this invention are those from natural sources. Cocoa provides a unique combination of xanthines, including theobromine and caffeine, and biogenic amines, and particularly phenylethylamine, in a form that is normally easily ingested and tolerated by the subject. In addition to the potentiating effect of the xanthines in cocoa, the MAO-inhibiting action of the phenylethylamine prolong the effects of serotonin, histamine and/or dopamine. Cocoa powder was originally included in preliminary formulations with neurotransmitter precursors to improve flavor and because its mood enhancing effects have appealed to people for centuries. An unexpected result was that the cocoa powder significantly potentiated the effects of the neurotransmitter precursors. This potentiating effect was determined by us to be produced by the naturally occurring xanthines and biogenic amines present in cocoa powder.

Infusions of caffeine from coffee beans and of caffeine and theophylline from tea leaves may be employed as a natural source of these xanthines, either in liquid form as coffee and tea, or in dried extract form, alone or, more inconveniently, in composition with the neurotransmitter precursor. Chocolate, guarana and other food sources may be employed.

The combinations of neurotransmitter precursors of this invention may be employed with an attendant synergistic effect, without concomitant administration of xanthine, and yet further potentiation may be achieved by administering the neurotransmitter precursor combinations with a xanthine. The neurotransmitter precursor combinations include histidine administered with tyrosine or with tryptophan and tyrosine followed by tryptophan after a time delay. Histidine does not compete with either tyrosine or tryptophan in crossing the blood-brain barrier so may be administered with either tyrosine or tryptophan at the same time and in the same composition.

Tyrosine and phenylalanine may be used conjointly with tryptophan in this invention with advantage but as they can inhibit passage of tryptophan across the blood-brain barrier, they are administered to the subject separately from the tryptophan, at time intervals of at least twenty minutes. Either the tryptophan or the tyrosine and/or phenylalanine may be administered before the other. Administered in this fashion to first permit take up of the phenylalanine and/or tyrosine from the blood stream, inhibition of tryptophan take-up is avoided and enhanced effect of the precursors is attained. Additionally, neurotransmitter balance is fostered by decreasing the total dose over time of any single neurotransmitter.

While it is not intended to be bound by any theory, the unexpected synergism found between these precursors may be at least partially explained by the different mechanisms mediated by their respective neurotransmiters in stimulating release of CRF and/or suppressing neuropeptide Y.

The dosage of each neurotransmitter precursor is in an amount sufficient to enhance synthesis of its respective neurotransmitter(s), to stimulate the release of CRF and thereby to suppress appetite in combined administration with the other neurotransmitter and or xanthines employed. The synergistic effect of these combinations will

permit appetite suppression at lower dosage levels of each of the neurotransmitter precursors than otherwise possible and desirably these lower dosage levels are employed to avoid possible side effects and particularly those now limiting the use of at tryptophan, including grogginess.

For tryptophan the desired single dose range is between 2.5 and 100 mg. with a typical dose of 45 mg. The desired dosage range of either phenylalanine or tyrosine is between 10 and 600 mg., with a typical dose of 500 mg. However, doses up to 700 mg. or even to 1 gram or higher, e.g. up to 3 grams, may be administered without undue risk of side effects. These amounts, equivalent to from .14 to 42.2 mg/kg, would be insufficient to suppress appetite if used alone. Histidine is desirably administered in a dosage range of 1 to 500 mg., with a typical dose of 30 mg. However somewhat higher doses, e.g. up to 1 gram, may be given, if tolerated by the subject. The dosage range for each precursor applies to combined administration of the precursor with another precursor, with a xanthine, or with both.

Where hydrolyzed proteins or proteins to be hydrolyzed in the gastrointestinal tract are employed as the source of tryptophan, the proteins should be in an amount to provide the tryptophan dosage levels of this invention as discussed above. Typically, this will be in a range of between around one half of a gram and 30 gm. The amount of enzyme employed may be 30 to 50 mg. per gram of protein. Insulin producing carbohydrates administered with the protein are desirably at dosage levels of from about one half gram to 5 grams.

Xanthines are employed in this invention in dosage ranges appropriate to promote release of neurotransmitters and to avoid undesired side effects. Theobromine and theophylline may each be administered in a dosage of from 1 mg. to 2 grams or higher. Caffeine may be administered in a dose of from 1 to 200 mg. or higher, if tolerated by the subject. Cocoa may be administered in a dose of 1 mg. to 2 grams or higher up to 20 grams for an appropriate dose of xanthines, with a preferred dose being 400 to 800 mg. Infusions such as tea or coffee may be employed, with one to two cups providing an appropriate dose. Somewhat higher doses of these xanthines may be employed with some subjects without undue discomfort.

The neurotransmitter precursors and neurotransmitter potentiators of this invention may be administered orally separately, or, for assurance of appropriate proportions and dosages as well as for convenience, they are administered together in the same composition. The dosage forms for administration separately or in the same composition may be any of the conventional forms, including capsules, capulets, chewable wafers, tablets, liquid suspensions, powders and the like. Xanthine dosages may take the form of chocolate preparations, cocoa drinks, infusions, e.g. coffee and tea and cola drinks containing caffeine. Hydrolyzed protein sources of tryptophan may be taken separately in tablet form, utilizing commercially available predigested protein tablets, such as LLP Concentrated Predigested Protein sold by Twin Laboratories, Inc., Ronkonkoma, New York containing aproximately 18 mg. of tryptophan per 1 gram tablet.

The compositions in the form of powders or liquids may be packaged in multiple dosage quantities with instructions to the user to extract therefrom for ingestion appropriate individual dosage amounts, e.g. a teaspoonful. However, the compositions are desirably prepared in discrete units, e.g. capsules, wafers etc., which each contain the appropriate dosage amounts of neurotransmitter precursors and/or neurotransmitter potentiators for a single dose as discussed above.

The compositions may include the usual carriers, fillers, excipients and adjuvants. Advantageously, they include soluble fiber, insoluble fiber, neurotransmitter precursors and the potentiating agents contained in cocoa powder. The inclusion of dietary fibers produces early satiety from volume distention and causes further appetite suppression by triggering the release of CCK. The appetite suppressing actions of the dietary fiber component further enhance the invention's neurotransmitter-related effects. They additionally may contain folic acid and vitamin B6 to enhance conversion of tryptophan to serotonin, tyrosine to dopamine and histidine to histamine, respectively.

The preferred amount of folic acid is 200 mcg per dose with a range of 1 - 800 mcg/dose. The preferred amount of vitamin B6 is 10 mg with a range of 1 - 50 mg/dose. Representative doses of soluble fibers are 100 mg to 1000 mg per dose. The best soluble fibers for producing appetite suppression are pectin fibers from apple or citrus. fruits. Representative doses of insoluble fibers are 100 mg to 1000 mg per dose.

A preferred embodiment utilizes insoluble fiber in the form of wheat bran for these formulations. Other suitable insoluble fibers include, but are not limited to cellulose, methyl-cellulose, chitosan, whey, whole wheat fiber, and other whole grain fiber. These concentration of insoluble fibers would be ineffective as appetite suppressants if given alone in these doses. The fibers must be premixed with water until barely wet and dried at low heat. The premix will result in a better gel and fat binding than the use of either type of fiber alone. Fiber which has not been premixed and heated to dryness will reduce the effectiveness of the formulations.

It is important in carrying out the invention to administer the dosages when the subject has an empty stomach, typically at least an hour after the subject has eaten in order to avoid undesirably slow uptake across the blood-brain barrier, due to competition with other amino acids from the ingested food. Administration may be repeated as desired, at intervals throughout the day.

The effects of the formations of this invention normally should be sufficiently potent that their effects can be experienced after the first dose. Their effectiveness can be detected by a given individual using a questionnaire to assess hunger and carbohydrate craving. This is in contradistinction to other appetite suppressants that require multiple doses or indirect methods such as weight loss to assess their effectiveness.

The various embodiments of the invention utilizing tryptophan, phenylalanine or tyrosine as the sole neurotransmitter precursor or combined with histidine, may be used alone. Advantageously, however, these tryptophan and phenylalanine or tyrosine formulations are given to a subject, but at different times, each to produce appetite suppression, but by different modalities. The phenylalanine or tyrosine-containing formulations are designed to potentiate the production and release of dopamine. Appetite suppression is achieved by the resulting activity of dopamine, and of histamine, if histidine is included. The phenylalanine or tyrosine-containing formulations emulate the effects of amphetamines, phentermine, ephedrine and pseudoephedrine. Tryptophan-containing formulations are designed to reduce appetite for 2-4 hours and are designed to potentiate the production and release of serotonin, and of histamine, if histidine is

included. Appetite suppression and reduced carbohydrate craving is achieved by the resulting activity of serotonin. The tryptophan-containing formulations emulate the effects of fenfluramine, d-fenfluramine and fluoxetine and are typically designed to reduce appetite for 1-4 hours and to reduce carbohydrate craving for 16-36 hours.

The tryptophan and phenylalanine or tyrosine formulations may be designed for use together in varying dosage schedules depending on individual needs. It is a preferred that each to be taken on an empty stomach. When used together in accordance with this invention, typically during the same day (24 hours), one is administered separately at least 20 minutes after the other. This is done to avoid competition of the precursors for entry across the blood brain barrier. Typically, the phenylalanine or tyrosine formulation is given before lunch to suppress appetite during the day and afternoon. The tryptophan formulation is given before dinner to decrease appetite and reduce carbohydrate craving at dinner and during the evening. Late afternoon and evening hours are the times of day when many over-weight people crave both food and carbohydrates. Alternately, The phenylalanine or tyrosine formulation can be administered at 10:00 a.m. and at 3:00 p.m. with the tryptophan formulation being administered at 11:00 a.m. and 4:00 p.m.. The dosage schedule allows these food supplements to emulate the effects of the prescription drugs phentermine, fenfluramine, and d-fenfluramine.

If an individual undergoes a fast to induce hunger, administration of tyrosine results in appetite suppression which begins 15 to 30 minutes after ingestion and continues for 2-4 hours. If hunger reappears, re-ingestion of the formulation results in suppression of hunger beginning 15-30 minutes after ingestion and continuing for 2-4 hours. Repeated administration of the tyrosine formulation results in repetitive suppression of appetite.

Administration of the tryptophan-containing formulation after a self-induced fast results in appetite suppression which begins 20-40 minutes after ingestion and continues for 2-4 hours. A reduction of carbohydrate craving begins approximately 30 minutes after ingestion of the tryptophan formulation and continues for 18-36 hours. If the tryptophan formulation is administered 30-90 minutes before the

tryptophan, the onset of the tryptophan formulation effects is reduced to 15-30 minutes.

Following Examples 1 through 7 illustrate formulations with tyrosine as the sole neurotransmitter precursor and formulations with tryptophan as the sole neurotransmitter precursor and use thereof independently and together. These examples also illustrate the use of various xanthines with the precursors and the use of hydrolyzed protein as the source of tryptophan.

## Example 1

A useful tyrosine formulation in one dose is tyrosine 295 mg, soluble fiber 125 mg, insoluble fiber 125 mg, cocoa 200 mg, vitamin B6 5 mg, and folic acid 100 mcg. A useful tryptophan combination per dose is soluble fiber 175 mg, insoluble fiber 175 mg, protein powder 100 mg, tryptophan 45 mg, vitamin B6 5 mg, and folic acid 100 mcg. Another useful tryptophan-containing formulation per dose is soluble fiber 175 mg, insoluble fiber 175 mg, predigested protein powder 2,000 mg, cocoa 250 mg, sugar 250 mg, vitamin B6 5 mg, and folic acid 100 mcg.. A preferred dosage of the combination is 2 capsules of tyrosine formulation before lunch, 2 capsules of the tyrosine formulation at 4:00 p.m., and 2 capsules of either of the tryptophan formulations 30 minutes before dinner. Another dosage schedule includes the tyrosine dose at 10:00 a.m. and 3:00 p.m. with tryptophan dose at 11:00 a.m. and 4:00 p.m. Other dosage schedules can be used.

## Example 2

This example illustrates the use of tyrosine as the sole neurotransmitter precursor, together with xanthines, for appetite suppression. A 53 year old male underwent a 10 hour fast to induce hunger. Two capsules of a tyrosine formulation were given each capsule containing soluble fiber in the form of apple pectin 175 mg, insoluble fiber in the form of bran fiber, tyrosine 295 mg, cocoa powder 200 mg, folic acid 100 mcg and vitamin B6 5 mg. The soluble and insoluble fibers had been premixed, wet and dried. The material had

been placed into capsules. The subject experienced an elimination of hunger that began 8 minutes after ingestion and lasted for 2.5 hours. A second ingestion of 2 capsules of the formulation reproduced the effect.

## Example 3

This example illustrate the use of tryptophan as the sole neurotransmitter precursor, together with xanthines, for appetite suppression and carbohydrate craving. A 44 year old male underwent a 10 hour fast to induce hunger. He then ingested 2 capsules of a tryptophan formulation each capsule containing 175 mg soluble fiber in the form of apple pectin and psyllium, 175 mg insoluble fiber in the form of bran fiber, 100 mg vegetable non-soy protein, 45 mg of tryptophan, 250 mg of cocoa powder, 5 mg of vitamin B6, and 100 mcg of folic acid. The individual's hunger began to dissipate in 30 minutes and was completely dissipated in 60 minutes. The ingestion of the formulation resulted in early satiety in the following meal. There was an abolition of carbohydrate craving which lasted for 24 hours. The onset of the appetite suppression following ingestion of the formulation was associated with mental grogginess that lasted for approximately 15 minutes.

## Example 4

This example illustrates the use of a tryptophan formulation utilizing predigested proteins as the tryptophan source. A 35 year old female underwent a 10 hour fast in order to induce hunger. She then ingested two capsules containing 175 mg soluble fiber in the form of apple pectin and psyllium, 175 mg insoluble fiber in the form of bran fiber, 2,000 mg of predigested protein in the form of predigested casein, 250 mg of cocoa powder, 250 mg sugar, 5 mg of vitamin B6, and 100 mcg of folic acid. She experienced a reduction of appetite and abolition of carbohydrate craving. There was no mental grogginess induced by this formulation.

Example 5

This example illustrates the use tyrosine and tryptophan of this invention together for appetite suppression, decreased carbohydrate craving and weight loss. The 53 year old male took 2 capsules of the formulation of daily at 10:00 am, 2 capsules of the formulation of Example 2 at 4:00 p.m. and 2 capsules of the formulation of Example 3 at 5:00 p.m.. This regimen was continued for 10 days. During the 10 day period, both of the formulations reduced appetite for 2-4 hours after each ingestion. Carbohydrate craving was reduced for 24 hours after ingestion of the tryptophan formulation. By the third day there appeared to be an enhanced effect in that the duration of action of the combined doses were prolonged. By the fifth day there was complete suppression of carbohydrate craving that lasted throughout the 10 day period. There were no observed side effect except for the 15 minutes of grogginess induced by the tryptophan formulation on days 1 and 2. For the first 2 days, the onset of the appetite suppression following ingestion of the tryptophan formulation was associated with mental grogginess that lasted for approximately 15 minutes. By the third day the grogginess effect was lost. The subject initially weighed 159 pounds and by the 10th day, his weight was reduced to 150 pounds.

Example 6

This example illustrates the use of tyrosine and tryptophan formulations of the invention together in an open label study of 5 subjects including 3 males and 2 females. Each subject took the tyrosine capsule of Example 2 at 10 AM and a typtophan capsule of Example 3 at 3:30 PM. All 5 subjects reported a decrease in hunger after either dose. All 5 Patients experienced a reduction of carbohydrate craving after the tryptophan capsule.

Example 7

This example illustrates the use of tyrosine and tryptophan formulations in a randomized double blind placebo controlled trial in 30 subjects. All 30 subjects underwent a 10 hour fast following which they completed a questionnaire to assess hunger on a 5 point scale and carbohydrate craving also measured on a 5 point scale. The subjects then ingested 2 of the capsules of Example 2 or placebo capsules at 10:00 a.m., followed by a questionnaire at 11:00 a.m.. The subjects again took the Example 2 capsule or placebo at 4:00 p.m. and the Example 3 capsule or placebo at 5:00 p.m. They completed questionnaires at 4:00, 5:00, 6:00 p.m. and at 10:00 a.m. the next morning. In the 15 placebo subjects, ingestion of the placebo was followed by an increase in the hunger index from 2.2 to 2.9 after the first dose of tyrosine, $p<0.03$. In the 15 subjects randomized to receive tyrosine, the hunger index fell from 3.1 to 2.4, $p<0.03$. Comparison of the active to placebo group showed a reduction of the hunger index with a high degree of significance, $p<0.01$. The carbohydrate craving index was also significantly reduced by the tryptophan dose, $p<.01$. In the active group, 85% of the subjects either reduced their feeling of hunger or cravings for carbohydrate while only 45% of the placebo group experienced either a reduction of hunger or cravings for carbohydrate, $p<0.03$.

Following examples 8 and 9 illustrate the formulation and use of histidine with xanthines, with histidine as the only neurotransmitter precursor.

## Example 8

A formulation of histidine and cocoa may be prepared by blending these two ingredients in powder form in a proportion of 3 parts histidine and 50 parts cocoa by weight. This product is then portioned into gelatin capsules so that each contains 30 mg. of histidine and 500 mg. cocoa. A one capsule dose of this formulation is best administered on an empty stomach, at least one or two hours after eating. Alternatively, the blended powder may be prepared in the form

of a chewable wafer sized to contain the same dose, by combining with the powder wheat bran, apple pectin and a sweetener.

Example 9

A formulation of histidine and caffeine may be prepared in the same manner as described in example 8 by blending in powder form histidine and caffeine in a proportion of 3 parts histidine and 10 parts caffeine by weight. Single dose capsules are then filled with this blend in an amount to each contain 30 mg. histidine and 100 mg. caffeine. This formulation is administered as in example 8.

Following examples 10 through 15 illustrate practice of the invention utilizing the combination of histidine with tyrosine and of histidine with tryptophan as neurotransmitter precursors, both with and without concomitant application of xanthines.

Example 10

A formulation of tryptophan and histidine may be prepared by blending these two ingredients in powder form in a proportion of 5 parts tryptophan and 3 parts histidine. This product is then portioned into gelatin capsules so that each contains 50 mg. of tryptophan 30 mg. histidine and the capsules are administered as in Example 8.

Example 11

A formulation as in Example 10 that contains caffeine in addition to tryptophan and histidine may prepared by blending in powder form 10 parts of caffeine with 5 parts tryptophan and 3 parts histidine. Gelatin capsules are filled with the powder blend so that each gelatin capsule contain 50 mg. of tryptophan 30 mg. histidine and 100 mg. of caffeine. This formulation is administered as in example 8.

Example 12

A formulation of tyrosine and histidine may be prepared by blending these two ingredients in powder form in a proportion of 50 parts tyrosine and 3 parts histidine. This product is then portioned into gelatin capsules so that each contains 500 mg. of tyrosine 30 mg. histidine and the capsules are administered as in Example 8.

Example 13

A formulation as in Example 12 that contains cocoa in addition to tyrosine and histidine may prepared by blending in powder form 50 parts of cocoa with 50 parts tyrosine and 3 parts histidine. Gelatin capsules are filled with the powder blend so that each gelatin capsule contain 500 mg. of tyrosine 30 mg. histidine and 500 mg. of cocoa. This formulation is administered as in example 8.

Example 14

A formulation of histidine with tryptophan in the form of enzyme hydrolyzed protein may be prepared as follows. Enzyme hydrolyzed milk protein (casein) in dry powder form containing approximately 18 mg. tryptophan per gram is blended with histidine in powder form in a proportion of 200 parts hydrolyzed protein and 3 parts histidine. This product is then portioned into gelatin capsules so that a single dose of 30 mg. histidine and 2 gm. of hydrolyzed milk protein, which provides approximately 32 mg. of tryptophan, is contained in three capsules. The capsules are administered as in Example 8.

Example 15

A formulation as in Example 14 that contains cocoa in addition to hydrolyzed milk protein and histidine may prepared by blending in powder form 50 parts of cocoa with 200 parts of the hydrolyzed milk protein and 3 parts histidine. Gelatin capsules are filled with the powder blend so that three capsules together contain a single dose of 30 mg. histidine, 2 gm. of hydrolyzed milk protein, which provides approximately 32 mg. of tryptophan, and 500 mg. of cocoa. This formulation is administered as in example 8.

Following examples 16 through 18 illustrate the practice of the invention utilizing unhydrolyzed protein, together with a proteolytic enzyme, as the source of the neurotransmitter precursor tryptophan, both with and without concomitant application of a xanthine and/or histidine as an additional neurotransmitter precursor.

## Example 16

This example illustrates the administration of tryptophan in accordance with this invention by giving to the subject orally unhydrolyzed protein together with a proteolytic enzyme which will hydrolyze the protein when it enters the gastrointestinal tract to release the tryptophan.

Specifically 10 grams of whey powder and approximately 40 mg. of papain powder were administered to a subject orally, on an empty stomach. With this high dosage, typtophan was released in the G.I. tract in an amount to induce appetite suppression, without the administration of xanthine. However, the subject experienced very pronounced grogginess that lasted for several hours.

Later, to the same subject, between 1 and 2 grams of whey powder, approximately 40 mg. of papain powder and 40 mg. of cocoa were administered, on an empty stomach. This formulation induced appetite suppression in the subject and no grogginess was experienced.

This procedure provides an easy mode of administering tryptophan using natural food sources together with xanthine to produce appetite suppression without undue grogginess.

Adminstration of this tryptophan source without xanthine, or a synergistic neurotransmitter precursor, required such a high dosage level to acheive appetite suppression that the side effects (grogginess) were unacceptable.

## Example 17

A formulation of cocoa with tryptophan in the form of unhydrolyzed protein together with a proteolytic enzyme to hydrolyze the protein in the G. I. tract may be prepared as follows. Whey in dry powder is blended with papain and cocoa in powder form in a proportion of 200 parts by weight of hydrolyzed protein, 4 parts papain and 50 parts cocoa. This product is then portioned into gelatin capsules so that each contains 500 mg. cocoa and 2 gm. of whey and 40 mg. papain. Hydrolysis of the whey in the gastrointestinal tract provides a dose of approximately 50 mg. of tryptophan. The capsules are administered as in Example 8.

## Example 18

A formulation is prepared and administered as in Example 17 but with the addition thereto of 3 parts histidine, thus additionally providing 30 mg. of histidine per capsule dosage.

As can be seen from the foregoing, the synergistic combinations of the invention allow reduced doses of the individual components to be used to achieve the desired effects and particularly of the neurotransmitter precursors. The reduced doses decrease the side effects caused by the large doses heretofore necessary to achieve the desired effects. Our invention allows appetite suppression and reduction of carbohydrate craving to be achieved at doses levels which are considered safe by regulatory authorities. Previous attempts to use certain of the components in isolation were either ineffective or required dosages which caused side effects.

The decreased dose of tryptophan, for example, allows reduction of carbohydrate craving without causing feelings of grogginess or safety concerns associated with higher doses. The reduced dose of tyrosine allows appetite suppression without the agitation and anxiety induced by amphetamines. The reduced dose of histidine reduces or eliminates potential side effects of histamine.

It is further seen that the combinations of the invention enable the use of naturally occurring substances thereby enhancing their regulatory approval and market acceptance.

Although the description above contains many specificities, these should not be construed as limiting the scope of the invention but as merely providing illustrations of some of the presently preferred embodiments of this invention. Various other embodiments and ramifications are possible within it's scope.

## Claims

1. A method for suppressing appetite in an animal subject which comprises concomitantly administering to the subject tryptophan in an amount effective to enhance synthesis of serotonin in the brain, in a dose of less than 100 mg, and a xanthine in an amount effective to enhance neural release of serotonin in the brain.

2. A method as in claim 1 and wherein the xanthine comprises caffeine.

3. A method as in claim 2 and wherein the caffeine administered is in a dose of between 1 and 200 mg.

4. A method as in claim 1 and wherein the xanthine comprises theobromine.

5. A method as in claim 4 and wherein the theobromine administered is in a dose of between 1 and 2,000 mg.

6. A method as in claim 1 and wherein the xanthine is in the form of cocoa.

7. A method as in claim 6 and wherein the cocoa administered is in a dose of between 1 and 2,000 mg.

8. A method as in claim 1 and wherein the tryptophan is administered in the form of enzyme hydrolyzed protein.

9. A method as in claim 8 and including the concomitant administration to the subject of a carbohydrate in an amount per dose sufficient to stimulate insulin production in the subject.

10. A method for suppressing appetite in an animal subject which comprises concomitantly administering to the subject a dopamine and norepinephrine precursor selected from phenylalanine and tyrosine in an amount effective to enhance synthesis of dopamine and norepinephrine in the brain, and a xanthine in an amount effective to increase neural release of dopamine and epinephrine in the brain.

11. A method as in claim 10 and including the administration of tryptophan to the subject in accordance with claim 1 at an interval of at

between 20 minutes and 24 hours from the time of administration of the dopamine and norepinephrine precursor.

12. A method as in claim 10 and wherein the dopamine and norepinephrine precursor administered is in a dose of between 10 and 700 mg.

13. A method as in claim 12 and wherein the dopamine and norepinephrine precursor administered is in a dose of less than 600 mg.

14. A method as in claim 10 and wherein the xanthine comprises caffeine.

15. A method as in claim 14 and wherein the caffeine administered is in a dose of between 1 and 200 mg.

16. A method as in claim 10 and wherein the xanthine comprises theobromine.

17. A method as in claim 16 and wherein the theobromine administered is in a dose of between 1 and 2,000 mg.

18. A method as in claim 10 and wherein the xanthine is in the form of cocoa.

19. A method as in claim 18 and wherein the cocoa administered is in a dose of between 1 mg. and 20 grams.

20. A method for suppressing appetite in an animal subject which comprises concomitantly administering to the subject histidine in an amount effective to enhance synthesis of histamine in the brain and a xanthine in an amount effective to increase neural release of histamine in the brain.

21. A method as in claim 20 and wherein the histidine dose administered is between 1 and 500 mg.

22. A method as in claim 20 and wherein the xanthine comprises caffeine.

23. A method as in claim 22 and wherein the caffeine administered is in a dose of between 1 and 200 mg.

24. A method as in claim 20 and wherein the xanthine comprises theobromine.

25. A method as in claim 24 and wherein the theobromine administered is in a dose of between 1 and 2,000 mg.

26. A method as in claim 20 and wherein the xanthine is in the form of cocoa.

27. A method as in claim 26 and wherein the cocoa administered is in a dose of between 1 mg. and 20 grams.

28. A method for suppressing appetite in an animal subject which comprises concomitantly administering to the subject the histamine precursor histidine in an amount effective to enhance synthesis of histamine in the brain and a second neurotranmitter precursor selected from tryptophan, phenylalanine and tyrosine in an amount effective to enhance synthesis in the brain of the neurotransmitters synthesized from the second precursor.

29. A method as in claim 28 and wherein the histidine dose administered is between 1 and 500 mg.

30. A method as in claim 28 and wherein the second precursor administered comprises tyrosine in a dose of between 1 and 600 mg.

31. A method as in claim 28 and wherein the second precursor administered comprises tryptophan in a dose of between 1 than 100 mg.

32. A method as in claim 28 and wherein the second precursor is tryptophan and the tryptophan is administered in the form of enzyme hydrolyzed protein.

33. A method as in claim 32 and including the concomitant administration to the subject of a carbohydrate in an amount per dose sufficient to stimulate insulin production in the subject.

34. A method as in claim 28 and including the concomitant administration to the subject of a xanthine in an amount effective to increase neural release in the brain of histamine and of the neurotransmitters synthesized from the second precursor.

35. A method as in claim 34 and wherein the xanthine comprises caffeine administered is in a dose of between 1 and 200 mg.

36. A method as in claim 34 and wherein the xanthine comprises theobromine administered is in a dose of between 1 and 2,000 mg.

37. A method as in claim 34 and wherein the xanthine is in the form of cocoa administered is in a dose of between 1 mg. and 20 grams.

38. A method for suppressing appetite in an animal subject which comprises concomitantly administering to the subject protein in an amount to comprise, upon enzyme hydrolysis thereof, sufficient tryptophan effective to enhance synthesis of serotonin in the brain, a proteolytic enzyme in an amount to hydrolyze the protein in the gastrointestinal tract to liberate the tryptophan and a xanthine in an amount effective to enhance neural release of serotonin in the brain.

39. A method as in claim 38 and wherein the xanthine comprises theobromine administered is in a dose of between 1 and 2,000 mg.

40. A method as in claim 38 and wherein the xanthine is in the form of cocoa.

41. A method for suppressing appetite in an animal subject which comprises concomitantly administering to the subject protein in an amount to comprise, upon enzyme hydrolysis thereof, sufficient tryptophan effective to enhance synthesis of serotonin in the brain, a proteolytic enzyme in an amount to hydrolyze the protein in the gastrointestinal tract to liberate the tryptophan and the histamine precursor histidine in an amount effective to enhance synthesis of histamine in the brain.

42. A method as in claim 41 and including the concomitant administration to the subject of a xanthine in an amount effective to increase neural release in the brain of histamine and of serotonin.

43. A composition for suppressing appetite in an animal subject, in unit dosage form, comprising tryptophan, in an amount between 1 mg and 100 mg per dose, and a xanthine in an amount effective to enhance neural release of serotonin in the brain of the subject.

44. A composition as in claim 43 and wherein the xanthine comprises theobromine in an amount of between 1 and 2,000 mg. per dose.

44. A composition as in claim 43 and wherein the xanthine comprises theobromine in an amount of between 1 and 2,000 mg. per dose.

45. A composition as in claim 43 and wherein the xanthine comprises caffeine in an amount of between 1 and 200 mg. per dose.

46. A composition as in claim 43 and wherein the xanthine is in the form of cocoa.

47. A composition as in claim 43 and wherein the tryptophan is present in the composition in the form of enzyme hydrolyzed protein.

48. A composition as in claim 47 and wherein the composition further comprises a carbohydrate, in an amount per dose sufficient to stimulate insulin production in a subject.

49. A composition for suppressing appetite in animal subject, in unit dosage form, comprising a dopamine and norepinephrine precursor, in an amount in an amount effective to enhance synthesis of dopamine and norepinephrine in the brain, and a xanthine in an amount effective to increase neural release of dopamine and epinephrine in the brain.

50. A composition as in claim 49 and wherein the xanthine comprises theobromine in an amount of between 1 and 2,000 mg. per dose.

51. A composition as in claim 49 and wherein the xanthine comprises caffeine in an amount of between 1 and 200 mg. per dose.

52. A composition as in claim 49 and wherein the xanthine is in the form of cocoa in the amount of between 1 and 2,000 mg. per dose.

53. A composition as in claim 49 and wherein the precursor is tyrosine in an amount per dose is between 1 and 600 mg.

54. A composition as in claim 53 and wherein the amount of tyrosine per dose is less than 500 mg.

55. A composition for suppressing appetite in animal subject, in unit dosage form, comprising histidine, in an amount in an amount effective to enhance synthesis of histamine in the brain, and a xanthine in an amount effective to increase neural release of histamine in the brain.

56. A composition as in claim 55 and wherein the xanthine comprises theobromine in an amount of between 1 and 2,000 mg. per dose.

57. A composition as in claim 55 and wherein the xanthine comprises caffeine in an amount of between 1 and 200 mg. per dose.

58. A composition as in claim 55 and wherein the xanthine is in the form of cocoa in the amount of between 1 mg. and 20 grams per dose.

59. A composition as in claim 55 and wherein the amount of histidine per dose is between 1 and 600 mg.

60. A composition for suppressing appetite in animal subject, in unit dosage form, comprising the histamine precursor histidine in an amount effective to enhance synthesis of histamine in the brain and a second neurotranmitter precursor selected from tryptophan, phenylalanine and tyrosine in an amount effective to enhance synthesis in the brain of the neurotransmitters synthesized from the second precursor.

61. A composition as in claim 60 and wherein the amount of histidine per dose is between 1 and 600 mg

62. A composition as in claim 60 and wherein the second precursor administered comprises tyrosine in a dose of between 1 and 600 mg.

63. A composition as in claim 60 and wherein the second precursor administered comprises tryptophan in a dose of between 1 and 100 mg.

64. A composition as in claim 60 and wherein the second precursor is tryptophan in the form of enzyme hydrolyzed protein.

65. A composition as in claim 64 and further including a carbohydrate in an amount per dose sufficient to stimulate insulin production in the subject.

66. A composition as in claim 60 and wherein the composition further comprises a xanthine in an amount effective to increase neural release in the brain of histamine and of the neurotransmitters synthesized from the second precursor.

67. A composition as in claim 66 and wherein the xanthine comprises theobromine in an amount of between 1 and 2,000 mg. per dose.

69. A composition for suppressing appetite in animal subject, in a dry unit dosage form, comprising powdered protein in an amount to comprise, upon enzyme hydrolysis thereof, sufficient tryptophan effective to enhance synthesis of serotonin in the brain and a proteolytic enzyme in an amount to hydrolyze the protein in the gastrointestinal tract to liberate the tryptophan and a xanthine in an amount effective to enhance neural release of serotonin in the brain.

70. A composition as in claim 69 and wherein the protein is in the amount of between about one half gram to 30 grams per unit dose and the enzyme is in the amount of between 30 and to 50 mg. per gram of protein.

71. A composition as in claim 70 and wherein the enzyme is papain.

72. A composition for suppressing appetite in animal subject, in a dry unit dosage form, comprising powdered protein in an amount to comprise, upon enzyme hydrolysis thereof, sufficient tryptophan effective to enhance synthesis of serotonin in the brain, a proteolytic enzyme in an amount to hydrolyze the protein in the gastrointestinal tract to liberate the tryptophan and the histamine precursor histidine in an amount effective to enhance synthesis of histamine in the brain.

73. A composition as in claim 72 and further comprising a xanthine in an amount effective to increase neural release in the brain of histamine and of serotonin.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/12408

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 31/52, 31/195, 35/12, 38/43
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According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | GB 2,004,183 A (THE PHARMACEUTICAL EXPORT PROMOTION COUNCIL) 28 March 1979, see the entire document. | 1-73 |
| Y | US 4,897,380 A (POLLACK et al) 30 January 1990, see the entire document. | 1-73 |
| Y | WO 91/10441 A1 (MEDGENIX GROUP S.A.) 25 July 1991, see the entire document. | 1-73 |
| Y | US 3,867,539 A (HENKIN) 18 February 1975, see the entire document. | 1-73 |
| Y | US 5,019,594 A (WURTMAN et al.) 28 May 1991, see the entire document. | 1-73 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
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| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 4,210,637 A (WURTMAN et al.) 01 July 1980, see the entire document. | 1-73 |

INTERNATIONAL SEARCH REPORT

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B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

CA, APS, MEDLINE BIOSIS, WPIDS:
search terms: (suppress? or reduc? or decreas?(5a)appetit?) and (tryptophan or histidine or tyrosine or phenylalanine or caffeine or xanthine)

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WO 2004/049833 A1

(54) Title: BEVERAGE WITH FOAM MAINTAINING SYSTEM

(57) Abstract: A beverage product comprises a container holding a liquid beverage component and sufficient nitrogen gas to give a gas pressure in the head space of at least 3.3 bar at 5°C, said liquid beverage comprising an organoleptically acceptable foam-maintaining system such that when the liquid beverage is poured from the container a foam is generated,the volume of which is maintained at greater than 80% of its initial volume for a period of at least 10, preferably at least 30 minutes. The initial volume of the foam may be less than 20% of the volume of the liquid beverage. The foam-maintaining system may comprise octenylsuccinic acid modified starch, and at least one surface active agent selected from the group consisting of acyl lactylate salts, proteins, protein hydrolysates, sucrose esters, and mixtures thereof.

# BEVERAGE WITH FOAM MAINTAINING SYSTEM

The present invention relates to beverage products and in particular of foaming beverage products.

There are many examples of foaming beverages which are produced by the use of inserts inside a pressurised can. In the United Kingdom many canned beers, stouts and lagers are sold in cans which contain a so-called "widget" which operates after the can is opened to give a head on the drink which is said to be comparable to the head produced on draught drinks dispensed in EP-A-360284, EP-A-577284, US-A-4996823, US-A-5009901,WO-A-9324384, WO-A-9504689. Examples of non-alcoholic pressurised beverages which are pressurised with nitrous oxide and/or carbon dioxide are described in US-A-6403137 and GB-A-2299978. Beverages that are packaged in a closed container in the presence of carbon dioxide or nitrous oxide and nitrogen are described in EP-A-745329 and EP-A-1034703. Foaming cappuccino coffee products can be made by adding to the coffee drink a creamer comprising protein, lipid and carrier and optionally a modified starch emulsifier or a surfactant as is described in US-A-6168819. Effervescent beverages which are intended to be dispensed directly into the mouth of the consumer are described in WO-A-02070371 and WO-A-02070372.

A first aspect of the present invention provides a beverage product comprising a container holding a liquid beverage component and sufficient nitrogen gas to give a gas pressure in the head space of at least 3.3 bar at 5°C, said liquid beverage

comprising an organoleptically acceptable foam-maintaining system such that when the liquid beverage is poured from the container a foam is generated, the volume of which is maintained at greater than 80% of its initial volume for a period of at least 10, preferably at least 30 minutes.

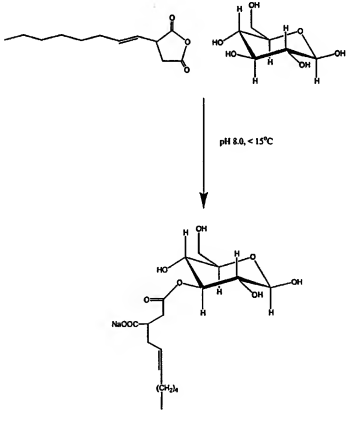
Preferably, the initial volume of the foam is less than 20% of the volume of the liquid beverage.

The container should be of sufficient strength that it can hold the pressure of the nitrogen gas inside it and should be impermeable to nitrogen gas. The container may be made of metal e.g., aluminium or steel, a plastic material for example polyethylene terephthalate or glass. The pressure of the gas in the head space within the container should preferably be in the range 3.3 to 6 bar at 5°C. The nitrogen gas may be introduced into the container in the form of liquid nitrogen. The term "nitrogen gas" as used herein is intended to include pure nitrogen gas or gas mixtures that are predominantly comprised of nitrogen. Preferably the nitrogen gas has purity of >97%.

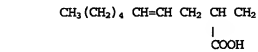
The liquid beverage component may be any consumable liquid. Examples of suitable liquids include optionally flavoured water, optionally flavoured milk, fruit flavoured liquids, tea or tea flavoured liquids, coffee or coffee flavoured liquids, chocolate, chocolate flavoured liquids, fruit smoothies or alcoholic or alcohol-free drinks such as cream liqueurs or cocktails.

In a preferred embodiment, the foam-maintaining system of the liquid beverage comprises 0.25 to 3.0% by weight of the liquid beverage component of octenylsuccinic acid modified starch, and at least one surface active agent selected from the group consisting of acyl lactylate salts, proteins, protein hydrolysates and sucrose esters and mixtures thereof.

The octenylsuccinic acid modified starch may be prepared by forming a covalent complex of a hydrophilic waxy maize starch with an octenylsuccinic acid moiety preferably its anhydride. The production of the octenylsuccinic acid modified starch is shown in the reaction scheme below.



Preferably the octenylsuccinic acid is a carboxy substituted undecenoic acid of formula



ie 3-carboxy-undec-5-enoic acid

The percentage molar substitution of octenylsuccinic acid groups may be in the range of 1.9 to 3%, preferably around 2.2%. The molecular weight of the octenylsuccinic acid modified starch is preferably in excess of 100,000 kDa.

The octenylsuccinic acid modified starch preferably comprises 0.75 to 1.5% by weight of the liquid beverage component. Suitable octenylsuccinate acid modified starch include those available from National Starch under the trade names Purity 2000, Purity 1773, Purity 539 and N-Creamer 46. A particularly preferred octenylsuccinic acid modified starch is available commercially from National Starch under the trade name N-Creamer 46

The viscosity of the liquid beverage component is preferably in the order of 1.5 to 100 $mPa.s^{-1}$, more preferably 30 to 60 $mPa.s^{-1}$ under low shear conditions (0.15 $s^{-1}$) at 5°C.

The acyl moiety of the acyl lactylate salt preferably contains 8 to 16 preferably 10 to 14 more preferably around 12 carbon atoms. The acyl lactylate salt may be a sodium or calcium salt.

Preferred acyl lactylate salts include calcium stearoyl lactylate and sodium stearoyl lactylate and mixtures thereof. The acyl lactylate salt preferably comprises 0.005 to 1 %, more preferably 0.01 to 0.5% by weight of the liquid beverage.

Suitable proteins and protein hydrolysates are those contained in or derived from milk for example caseinate salts such as sodium caseinate, whey protein isolates or milk protein hydrolysates. The protein and/or protein hydrolysate preferably comprises 0.01 to 0.5 %, more preferably 0.1 to 0.3% by weight of the liquid beverage.

Sucrose esters are esters prepared from sucrose and fatty acids derived from edible fats and oils. Preferred sucrose esters are predominantly monoesters. The fatty acid moiety preferably contains 8 to 16 carbon atoms. Suitable fatty acids include caprylic acid, lauric acid, myristic acid, palmitic acid, stearic acid and mixtures thereof. Suitable sucrose esters are commercially available from Ryoto under the trade names P-1570 (70% monoester with fatty acids derived from vegetable oils containing 70% palmitic acid) and M-1695 (80% monoester with fatty acids derived from vegetable oils containing 95% myristic acid). The sucrose ester preferably comprises 0.02 to 0.4%, more preferably 0.05 to 0.3% of the liquid beverage.

In preferred beverage products of the present invention the surface active agent comprises an acyl lactylate salt either alone or in combination with a sucrose ester, a protein or a protein hydrolysate.

The surface tension of the liquid beverage component should be in the order of 65 to 20 $N.m^{-2}$, more preferably 40 to 20 $N.m^{-2}$.

The beverages of the present invention may contain additional constituents. Examples of suitable additional constituents include:-

(a) sweeteners for example natural sweeteners such as sugars (glucose, fructose, sucrose or corn syrup) or artificial sweeteners such as saccharin, aspartame or acesulfam.
(b) Preservatives for example benzoate or sorbate salts
(c) Antioxidants for example ascorbic acid or salts thereof or tocopherols
(d) Flavour enhancers for example maltol
(e) Flavourings for example fruit flavours or vanilla
(f) pH adjusting agents for example sodium bicarbonate
(g) viscosity adjusting agents for example propylene glycol alginate, carboxymethyl cellulose, high methoxy pectin and/or gums such as guar gum

A second aspect of the present invention provides a method of making a beverage product comprising a container holding a liquid beverage component and nitrogen gas, said liquid beverage comprising an organoleptically acceptable foam-maintaining system, said method comprising the steps of:-

incorporating the organoleptically acceptable foam-maintaining system into the liquid beverage,

placing the liquid beverage into the container,

adding sufficient liquid nitrogen to the container to provide a head space pressure of 3.3 to 6 bar at 5°C in the container after sealing, and

sealing the container.

A third aspect of the present invention provides a method of making a beverage product comprising a container holding a liquid beverage component and nitrogen gas, said liquid beverage comprising octenylsuccinic acid modified starch, and at least one surface active agent selected from the group consisting of acyl lactylate salts, proteins, protein hydrolysates and sucrose esters and mixtures thereof, said method comprising the steps of:-

incorporating the octenylsuccinic acid modified starch and the at least one surface active agent into the liquid beverage,

placing the liquid beverage into the container,

adding sufficient liquid nitrogen to the container to provide a head space pressure of 3.3 to 6 bar at 5°C in the container after sealing, and

sealing the container.

The contents of the sealed container may be sterilised after sealing by the application of heat for example by pasteurisation or retorting. Alternatively the product may be subjected to microfiltration or may be filled aseptically.

The present invention provides a beverage which is retained under pressure inside the container before the container is

opened but when the nitrogen becomes supersaturated after the container is opened, comes out of solution and forms a stable foam on top of the liquid beverage. In the beverage products of the present invention no widget is required to achieve this. The presence of the foam on top of the dispensed liquid beverage provides a pleasant drinking experience ( eg a pleasant taste and creamy mouthfeel) to the consumer as the beverage is consumed. The product may be consumed straight from the container but is preferably poured into a drinking vessel for example a glass before consumption.

The invention will be illustrated by the following non-limiting examples

Example 1

A milked tea beverage was made as described below.

1) Black tea leaf tea (0.6kg) was extracted with water (18L) at 90 ± 1°C for 3 minutes. The infusion was then passed through a 20 mesh screen, followed by a 150 mesh screen and cooled to 20-30°C. The infusion was then clarified using a centrifuge.
2) Sugar (5.5kg) was dissolved in hot water (6L), sterilised by UV treatment and added to the tea extract.
3) UHT-treated skimmed milk (10.6kg) was added to the resulting mixture
4) Sodium ascorbate (0.05kg) was dissolved in water (2L) and the solution added to the mixture.

5) Water was added to a volume of 100L

6) The mixture was homogenised at 60-70°C @ 200 $kgf.cm^{-2}$ and heated to 85°C

7) Skimmed milk powder (1.106kg) was added and mixed at 13,500rpm for 2 minutes.

8) Sodium stearoyl lactylate (0.5kg) was added and mixed at 13,500 rpm for 2 minutes

9) N-Creamer 46 modified starch (1kg - ex National Starch) was added and mixed at 13,500 rpm for 2 minutes at 65°C.

10) The resulting solution was cooled to 10°C and maltol (0.03kg) was added The mixture (<295ml) was filled into standard 330ml beverage cans and sufficient liquid nitrogen was injected into the cans to give a head space pressure of 3.5 ± 0.2 bar at 5°C. The cans were then rapidly sealed.

11) The sealed cans were then retorted at 140°C for 5 minutes

The resulting beverage contained the following constituents

| Constituent | Amount |
|---|---|
| Water | to 100% |
| UHT milk | 10.60 % |
| Granulated sugar | 5.5% |
| Tea solids | 0.2% |
| Skimmed milk powder | 1.16% |
| Tea flavour mix 06 | 0.16% |
| Sodium ascorbate | 0.05% |
| Maltol | 0.03% |
| N-Creamer 46 | 1.0% |
| Sodium stearoyl lactylate | 0.5% |

Example 2

A tea beverage was made as described below.

(1) Leaf tea (0.65 kg) was extracted with water (90L) at 90 ± 1°C for 5 min. The infusion was then passed through 4 layers of muslin cloth and the temperature was held at 70°C.

(2) Sodium bicarbonate (0.01 kg) was dissolved in the filtered infusion

(3) Sugar (3.9 kg) was dissolved in the infusion at 70°C by stirring gently for 1 minute.

(4) Caramel (0.1kg) was added to the infusion at 70°C

(5) Sodium stearoyl lactylate (0.5kg) added and mixed at 13,500 rpm for 2 minutes

(6) N-Creamer 46 starch (1kg) added and mixed at 13,500 rpm for 2 minutes at 65°C

(7) The resulting solution was cooled to 10°C

(8) Maltol (0.03kg) was added

(9) Sodium ascorbate (0.05kg) was dissolved in water (2L) and added to the mixture

(10) Tea aroma concentrate (2 kg) was added and the mixture was made up to 100l with water.

(11) The beverage mixture (<295ml) was filled into standard 330ml aluminium cans

(12) Liquid nitrogen was injected in order to give a head space pressure of 3.5 ± 0.2 bar at 5°C and the cans were sealed rapidly.

(13) The mixture was then retorted at 140°C for 5 minutes.

The resulting beverage contained the following constituents

| Constituents | Amount |
|---|---|
| Water | to 100% |
| Tea solids | 0.21% |
| Sugar | 3.9% |
| Tea aroma concentrate | 2.0% |
| Sodium ascorbate | 0.05% |
| Sodium bicarbonate | 0.01% |
| N-creamer 46 | 1.0% |
| Sodium stearoyl lactylate | 0.5% |
| Maltol | 0.03% |
| Caramel | 0.1% |

Example 3

An Irish coffee-type beverage was made as described below.

(1) Water (81.2 kg) was heated to 75°C

(2) Sugar (3.5kg) was added and completely dissolved at 70°C

(3) A mixture of sodium stearoyl lactylate (0.05kg), calcium stearoyl lactylate (0.05kg) and sucrose monoesters (0.2kg) was added and mixed at 13,500 rpm at 70°C

(4) Skim milk powder (1.0kg) was added and mixed at 13,500 rpm at 70°C

(5) N-Creamer 46 (1.0 kg) was added and mixed at 13,500 rpm at 70°C

(6) Instant coffee powder (0.8 kg) was added and dissolved at 60°C

(7) The mixture was cooled to ambient temperature and whiskey (12.2kg) was added

(8) The beverage (<295ml) was placed in a standard aluminium can (330ml) and sufficient liquid nitrogen was added to give a head pressure of 3.5 ± 0.2 bar at 5°C and can was sealed rapidly. Note. The product was filled and nitrogenated under aseptic conditions.

The resulting beverage contained the following constituents

| Constituent | Amount |
|---|---|
| water | to 100% |
| sugar | 3.50% |
| Sodium stearoyl lactylate | 0.05% |
| Calcium stearoyl lactylate | 0.05% |
| Sucrose monoesters | 0.20% |
| Skimmed milk powder | 1.0% |
| N-creamer 46 | 1.0% |
| coffee | 0.80% |
| whiskey | 12.20% |

Example 4

A raspberry flavoured smoothie type beverage was made as described below.

(1) Water (90 kg) is heated to 75°C

(2) Sugar (4 kg) is added and completely dissolved at 70°C

(3) Sodium stearoyl lactylate (0.5 kg) is added and mixed at 13,500 rpm at 70°C

(4) Skim milk powder (1 kg) is added and mixed at 13,500 rpm at 70°C

(5) N-Creamer 46 (1 kg) is added and mixed at 13,500 rpm at 70°C

(6) pH of solution is increased to pH 7.0 using 1.0M NaoH

(7) Cooled to ambient temperature and raspberry juice(10 kg) is added. The pH of the solution is maintained at pH 6.5 with the addition of 1.0M NaOH

(8) The beverage (<295ml) was placed in a standard aluminium can (330mL).

(9) Sufficient liquid nitrogen was added to give a head pressure of 4 bar at 5°C and the can was sealed rapidly.

(10) The can was retorted at 121°C for 5min.

The resulting beverage contained the following constituents

| Constituent | Amount |
|---|---|
| Water | to 100% |
| Raspberry juice | 10% |
| Sugar | 4% |
| N-creamer 46 | 1% |
| Sodium stearyl lactylate | 0.5% |
| Skim milk powder | 1% |
| Vanilla | 0.05% |

Example 5

A milked tea beverage was made as described below.

(1) Black tea leaf tea (0.6kg) was extracted with water (80L) at 90 ± 1°C for 3 minutes. The infusion was then passed through a 20 mesh screen, followed by a 150 mesh screen and cooled to 20-30°C. The infusion was then clarified using a centrifuge.

(2) Sugar (5.5kg) was dissolved in hot water (6L), sterilised by UV treatment and added to the tea extract.

(3) UHT-treated skimmed milk (10.6kg) was added to the resulting mixture

(4) Sodium ascorbate (0.05kg) was dissolved in water (2L) and the solution added to the mixture.

(5) Water was added to a volume of 90L

(6) The mixture was homogenised at 60-70°C at 19.6kPa. [200 $kgf.cm^{-2}$] and heated to 85°C

(7) Skimmed milk powder (1kg) was added and mixed at 13,500rpm for 2 minutes.

(8) Sodium stearoyl lactylate (0.06kg) was added and mixed at 13,500 rpm for 2 minutes

(9) N-Creamer 46 modified starch (1.25kg - ex National Starch) was added and mixed at 13,500 rpm for 2 minutes at 65°C.

(10) 0.2kg of milk protein hydrolysate (Hyfoama, ex. Quest) and dissolved thoroughly at 65°C

(11) The resulting solution was cooled to 10°C and maltol (0.03kg) was added.

(12) The solution was made to 100L with water.

(13) The mixture (<295ml) was filled into standard 330ml beverage cans and sufficient liquid nitrogen was injected into the cans to give a head space pressure of 3.5 ± 0.2 bar at 5°C. The cans were then rapidly sealed.

(14) The sealed cans were then retorted at 140oC for 5 minutes

The resulting beverage contained the following constituents

| Constituent | % solids |
|---|---|
| Water | to 100% |
| UHT milk | 10.60 % |
| Granulated sugar | 5.5% |
| Sucrose esters (P1570) | 0.1% |
| Hydrolysed milk protein (Hyfoama DS, Quest) | 0.2% |
| Tea solids | 0.2% |
| Skimmed milk powder | 1% |
| Tea flavour mix 06 | 0.16% |
| Sodium ascorbate | 0.05% |
| Maltol | 0.03% |
| N-Creamer 46 | 1.25% |
| Sodium stearoyl lactylate | 0.06% |

Comparative Examples A and B

In a similar way to that described above in Example 3, samples of beverages which had the same constituents as Example 3 were prepared except that Comparative Example A did not contain any surface active agents and comparative Example B did not contain any octenylsuccinic acid modified starch. The products were stored at 5°C for 3 hours and were then opened and poured into a graduated glass vessel. The amount of foam generated as the beverage was poured was determined from the graduations on the glass vessel. The amount of foam expressed as a percentage of the volume of foam present immediately after pouring was determined periodically for the beverage of Example 3 and for both of the Comparative Examples A and B. The results are shown in the Table below

| | Example 3 | Example A | Example B |
|---|---|---|---|
| Foam volume | 6.34% | 6.66% | 7.93% |
| Time (minutes) | | Foam volume as % of volume at $t_0$ | |
| 2.5 | 100 | 100 | 100 |
| 5 | 100 | 75 | 100 |
| 10 | 100 | 50 | 60 |
| 15 | 100 | 50 | 44 |
| 20 | 100 | 50 | 20 |
| 30 | 95 | 40 | 20 |
| 40 | 90 | 35 | 12 |
| 60 | 90 | 25 | 8 |

From the Table it can be seen that the foam generated from Example 3 lasts considerably longer than the foam generated from either of the Comparative Examples.

CLAIMS

1) A beverage product comprising a container holding a liquid beverage component and sufficient nitrogen gas to give a gas pressure in the head space of at least 3.3 bar at 5°C, said liquid beverage comprising an organoleptically acceptable foam-maintaining system such that when the liquid beverage is poured from the container a foam is generated, the volume of which is maintained at greater than 80% of its initial volume for a period of at least 10 minutes.

2) A beverage product as claimed in claim 1 wherein the volume of the foam is maintained at greater than 80% of its initial volume for a period of at least 30 minutes.

3) A beverage product as claimed in claim 1 wherein the initial volume of the foam is less than 20% of the volume of the liquid beverage.

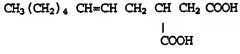
4) A beverage product as claimed in claim 1 wherein the pressure of nitrogen in the head space of the container is in the range 3.3 to 6 bar at 5°C.

5) A beverage product as claimed in any one of claims 1 to 4 wherein the foam maintaining system of the liquid beverage comprising 0.25 to 3.0% by weight of the liquid beverage component of octenylsuccinic acid modified starch, and at least one surface active agent selected from the group

consisting of acyl lactylate salts, proteins, protein hydrolysates and sucrose esters and mixtures thereof.

6) A beverage product as claimed in claim 5 wherein the octenylsuccinic acid modified starch is prepared by forming a covalent complex of a hydrophilic waxy maize starch with an octenylsuccinic acid moiety

7) A beverage product as claimed in claim 5 or claim 6 wherein the octenylsuccinic acid is a carboxy substituted undecenoic acid of formula



8) A beverage product as claimed in any one of claims 5 to 7 wherein the percentage molar substitution of octenylsuccinic acid groups in the range of 1.9 to 3%.

9) A beverage product as claimed in any one of claims 5 to 8 wherein molecular weight of the octenylsuccinic acid modified starch is in excess of 100,000 kDa.

10) A beverage product as claimed in any one of claims 5 to 9 wherein the acyl moiety of the acyl lactylate salt contains 8 to 16 carbon atoms.

11) A beverage product as claimed in any one of claims 5 to 10 wherein the acyl lactylate salt is a sodium or calcium salt

12) A beverage product as claimed in any one of claims 5 to 11 wherein the acyl lactylate salt is calcium stearoyl lactylate, sodium stearoyl lactylate or mixtures thereof.

13) A beverage product as claimed in any one of claims 5 to 12 wherein the acyl lactylate salt comprises 0.005 to 1% by weight of the liquid beverage.

14) A beverage product as claimed in any one of claims 5 to 13 wherein the proteins and protein hydrolysates are those contained in or derived from milk

15) A beverage product as claimed in any one of claims 5 to 14 wherein the proteins and protein hydrolysates are selected from sodium caseinate, whey protein isolates or milk protein hydrolysates

16) A beverage product as claimed in any one of claims 5 to 15 wherein the sucrose ester is predominantly a monoester.

17) A beverage product as claimed in any one of claims 5 to 16 wherein the sucrose ester is prepared from sucrose and fatty acids derived from edible fats and oils, said fatty acids containing 8 to 16 carbon atoms

18) A beverage product as claimed in claim 17 wherein the fatty acid is caprylic acid, lauric acid, myristic acid, palmitic acid, stearic acid or mixtures thereof

19) A beverage product as claimed in any one of claims 5 to 18 wherein the sucrose ester comprises 0.02 to 0.4% by weight of the liquid beverage.

20) A method of making a beverage product comprising a container holding a liquid beverage component and nitrogen gas, said liquid beverage comprising an organoleptically acceptable foam-maintaining system, said method comprising the steps of:-

incorporating the organoleptically acceptable foam-maintaining system into the liquid beverage,

placing the liquid beverage into the container,

adding sufficient liquid ntrogen to the container to provide a head space pressure of 3.3 to 6 bar at 5°C in the container after sealing, and

sealing the container.

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/12606

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A23L2/40 A23L2/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A23L B65B B01F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 17 February 2004 | 02/03/2004 |

| Name and mailing address of the ISA | Authorized officer |
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| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Groh, B |

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